Neural Substrates of Experience in Caenorhabditis elegans Olfactory Learning

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A dissertation presented

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Abstract

One essential function of the nervous system is to modulate behavioral response based on experience. In the past decades, increasing amount of studies has characterized the mechanisms underlying experience-dependent modulation of neural circuits. However, it is not entirely clear how the nervous system translates experience into a modulatory signal. The over-arching goal of my thesis work is to contribute to our understanding of this important neuroscience question.

I address this question by using a form of aversive olfactory learning in *Caenorhabditis elegans.* While *C. elegans* feeds on bacteria, it is susceptible to infectious bacteria. Ingestion of these pathogens makes the animal ill and leads to a slow death over time. Previous work in my advisor's laboratory has shown that *C. elegans* learns to avoid the smell of the infectious bacteria after ingestion and this experience-dependent olfactory plasticity depends on the serotonin signaling from a pair of serotonergic neurons called ADF and the serotonin signaling is up-regulated by pathogen stress stimuli. ADF acts in a neuronal network to play an essential role in regulating the aversive olfactory learning. Because serotonin signal responds to a variety of environmental stresses in many animals, including mammals, I use the *C. elegans* aversive olfactory training as a model to characterize the molecular and cellular mechanisms underlying the regulation of serotonin signaling by aversive experience.

At the level of neuronal physiology, I show that the calcium transients in the serotonergic neurons ADF are enhanced by the aversive experience with the pathogenic bacteria PA14. The aversive experience also increases the transcription of *tph-1*, which is the rate-limiting enzyme of serotonin synthesis. I show that this transcriptional regulation is dependent on the neuronal activity of ADF. At the molecular level, I demonstrate that UNC-43, the *C. elegans* CaMKII homolog, is activated by the pathogen training and acts in ADF neurons cell-autonomously to enhance ADF calcium signal and *tph-1* transcription. The activity of UNC-43 in ADF neurons is also essential for the learned avoidance against the odor of PA14. At the circuit level, EGL-30, the *C. elegans* Gq homolog mediates the sensory responses of AWB and AWC olfactory neurons to up-regulate *tph-1* transcription in ADF neurons. Altogether, a small neuronal network engages the function of a conserved molecular pathway of CaMKII and Gq to mediate serotonergic response to aversive experience with pathogenic infection.

I further explore two related questions. By analyzing PA14 mutants that are defective in a set of virulence factors, I probe which components in PA14 pathogenesis induce *tph-1* transcription. Because enhanced ADF serotonin signaling has been implicated in a variety of behaviors, I seek the resulting effects of the *tph-1* transcriptional regulation in circuit function. I demonstrate that transcriptional regulation of *tph-1* contributes to the decreased preference for pathogenic bacteria. These preliminary results point to future directions to examine the nature of the aversive sensory experience.

In summary, this thesis uncovers novel molecular and cellular mechanisms for regulation of serotonergic system in response to aversive experience. Because serotonin signals regulate stress

response and experience-dependent neural plasticity in both invertebrates and vertebrates, our findings are expected to generate general impacts on the research in the field.

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Acknowledgments

Looking back, graduate school was a sheer adventure, aside from how risky it felt going to a graduate school in a foreign country. Fortunately, I had so many people who supported me throughout my graduate study. Without them, the work presented in this thesis would not have been possible.

During my junior year of college, I hoped that I would become a graduate student studying the nervous system. My college consisted of six campuses. I stayed in the engineering campus, located in the west side of the West Lake. I biked half an hour, twice a week, to take neuroscience class in the medical school campus, in the east side of the West Lake.

I was incredibly lucky to have met a professor who not only encouraged me, an engineering undergraduate student, to pursue the dream of studying the fundamental neuroscience but did all she could to help me realize it. That professor is my advisor Professor Yun Zhang. I am grateful for what she has done to transform me from an engineering student to a neuroscience researcher. Very early on in my graduate studies, Yun taught me the techniques of molecular biology and microinjection, hand in hand. (I was very nervous when she stood beside me.) But unfortunately, it took me a lot of trials to make these experiments finally work. Today, after teaching many people these techniques, I now understand how patient she was with me at the time. When it took me more effort to make the calcium imaging system work than anything I had ever done before, and obtaining the expression of Cameleon in the ADF neurons seemed impossible at the beginning, I wanted to hide. But Yun would stand behind me and say, “You do not escape.

Doing research is not easy. Don't get bogged down by these experiment details. Even when you are in a bind, you don't forget the big picture of science. And that big picture is always fun." Working hard when things are going well is not a big deal. Yun's constant curiosity in science and perseverance in research taught me that, to make the dream come true, you have to keep working hard when things are not going well.

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These last few sentences have been saved for my husband, Dr. Meng Su. He is an extraordinary person. When enjoying physics, he does not need food or sleep. I thank him for giving me the feeling of safety when Perkins Hall was quiet and empty during the holidays. I thank him not only for sharing my happiness but also for tolerating all of my worries and frustrations.

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college education. In my memory, she was always busy taking care of me and reading books. She was able to obtain her bachelor's degree when I was in middle school. My father Jianlin Qin is curious about every machine in the world. It is impressive that he can make every piece of broken machine start up and work again. My family has taught me that life is tough, but one has to look for the beauty in it. Respect ordinary people and always keep compassion, and you will find it.

This thesis is dedicated to

my father Jianlin Qin and my mother Liping Zhang

Preface

“It has generally been the custom of writers on Natural History to take the habits and instincts of animals as fixed points, and to consider their structure and organization, as specially adapted, to be in accordance with these. This assumption is however an arbitrary one, and has the bad effect of stifling inquiry into the nature and causes of "instincts and habits," treating them as directly due to a "first cause," and therefore, incomprehensible to us. I believe that a careful consideration of the structure of a species, and of the peculiar physical and organic conditions by which it is surrounded, or has been surrounded in past ages, will often, as in this case, throw much light on the origin of its habits and instincts. These again, combined with changes in external conditions, react upon structure, and by means of "variation" and "natural selection", both are kept in harmony.”

- *Wallace, Alfred Russel (1869). The Malay Archipelago.*

Chapter 1

Introduction

Serotonin is a biogenic monoamine neurotransmitter that carries a broad range of behavioral and physiological functions in humans, including regulation of appetite, mood, sleep, reproduction and certain cognitive functions (Berger, Gray et al. 2009). The dysregulation of serotonin in the central nervous system is associated with mental disorders such as depression, anxiety and posttraumatic stress disorder (PSD) (Lesch, Bengel et al. 1996). Serotonergic neurons in the human central nervous system are located in the nine raphe nuclei in the brainstem (Bear, Connors et al. 2007; Berger, Gray et al. 2009). These neurons send axons and release serotonin to cerebellum, spinal cord and regions throughout the central nervous system (Berger, Gray et al. 2009).

Serotonin was identified from two tissues independently. An Italian scientist, Vittorio Erspamer, first extracted it in 1935 from a type of enteroendocrine cells called enterochromaffin cells. Erspamer showed that the unknown amine could contract intestines and he named it

"enteramine" (Renda 2000). 13 years later, in 1948, three American scientists, Maurice Rapport, Arda Green and Irvine Page, found a substance from blood serum that could constrict the blood vessel and they named it "serotonin" (Rapport, Green et al. 1948). 4 years after this discovery, it was found that "enteramine" and "serotonin" were the same monoamine (Feldberg and Toh 1953).

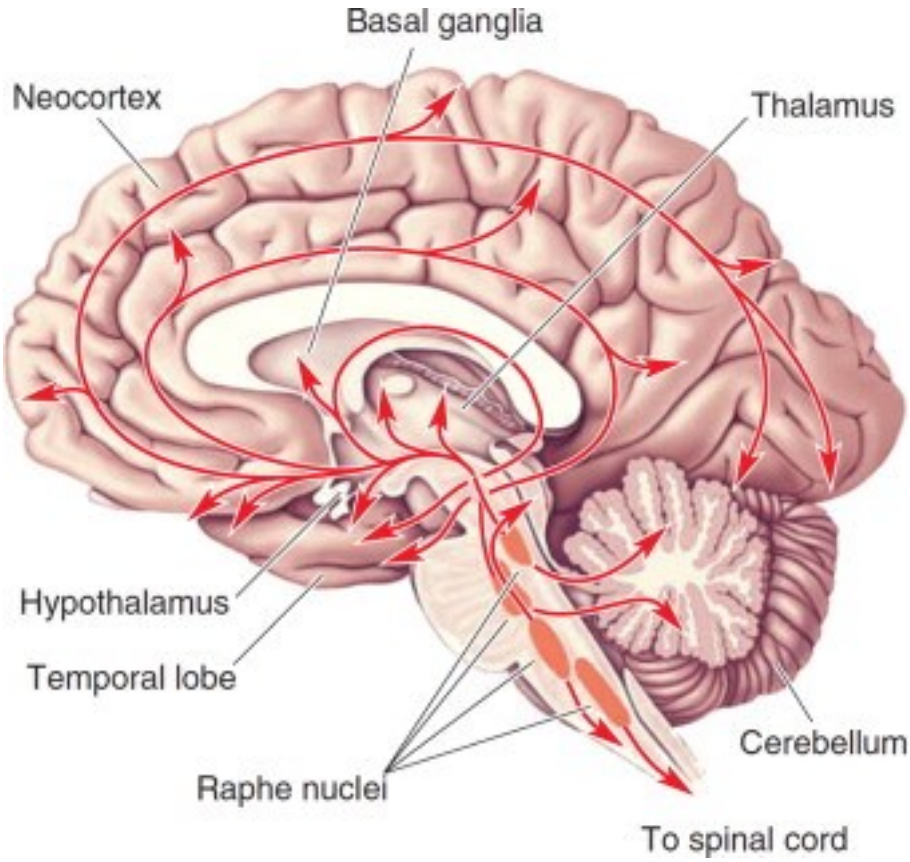


Figure 1.1 The serotonergic system in the human central nervous system (Bear, Connors et al. 2007).

Serotonergic neurons arise from the raphe nuclei clustered along the midline of the brain stem and project extensively to all levels of the central nervous system.

In the human central nervous system, serotonin is released by neurons located in the nine raphe nuclei in the brain stem. These neurons send axons to broad brain areas (Figure 1.1). In these neurons, serotonin is synthesized from tryptophan, which is an amino acid transported into the brain through the blood brain barrier. Tryptophan is first hydrolyzed to 5-HTP by tryptophan hydroxylase (TPH) and 5-HTP is then decarboxylated to serotonin (5-HT) by amino acid decarboxylase (Bear, Connors et al. 2007; Berumen, Rodriguez et al. 2012). The reaction catalyzed by TPH is the rate-limiting step in serotonin biosynthesis. There are two isoforms of TPH in mammals. TPH1 is expressed in the periphery (the enterochromaffin cells in the gut) and the pineal gland, and TPH2 is expressed in the brain and enteric neurons (Cote, Thevenot et al. 2003; Walther and Bader 2003; Walther, Peter et al. 2003; Zhang, Beaulieu et al. 2004; Zill, Buttner et al. 2004; McKinney, Knappskog et al. 2005) (Figure 1.2). In the central nervous system, serotonin has three functional modes. The first mode is to act as a classical neurotransmitter. After released from the presynaptic site, serotonin binds to its receptors at the postsynaptic site and then activates or inhibits the postsynaptic neurons directly. The second mode is to act as a neuromodulator. Instead of exerting direct effect, a neuromodulator modulates the response of postsynaptic neurons or the release of the primary neurotransmitter from the presynaptic neurons. The third mode is to function as a neurohormone. Instead of functioning in a synaptic cleft, serotonin can diffuse into a relative large brain area and exerts a broad modulatory function (Weiger 1997). In all these three modes, serotonin signals through seven families of serotonin receptors. Except for 5-HT_3, which is a ligand-gated Na^+ and K^+ cation channel, all other serotonin receptors are G protein coupled receptors (Berumen, Rodriguez et al. 2012). Finally, serotonin can be either transported back to the synaptic terminal by serotonin transporter (SERT) or degraded to 5-Hydroxyindoleacetic acid (5-HIAA) by monoamine

oxidases (MAO) in the mitochondria. Both SERT and MAO are the targets for treating psychiatric disorders (which will be discussed later) (Buller, Wixey et al. 2012). In the pineal gland, serotonin is further converted to melatonin, which is a hormone regulated by circadian clock (Chattoraj, Liu et al. 2009).

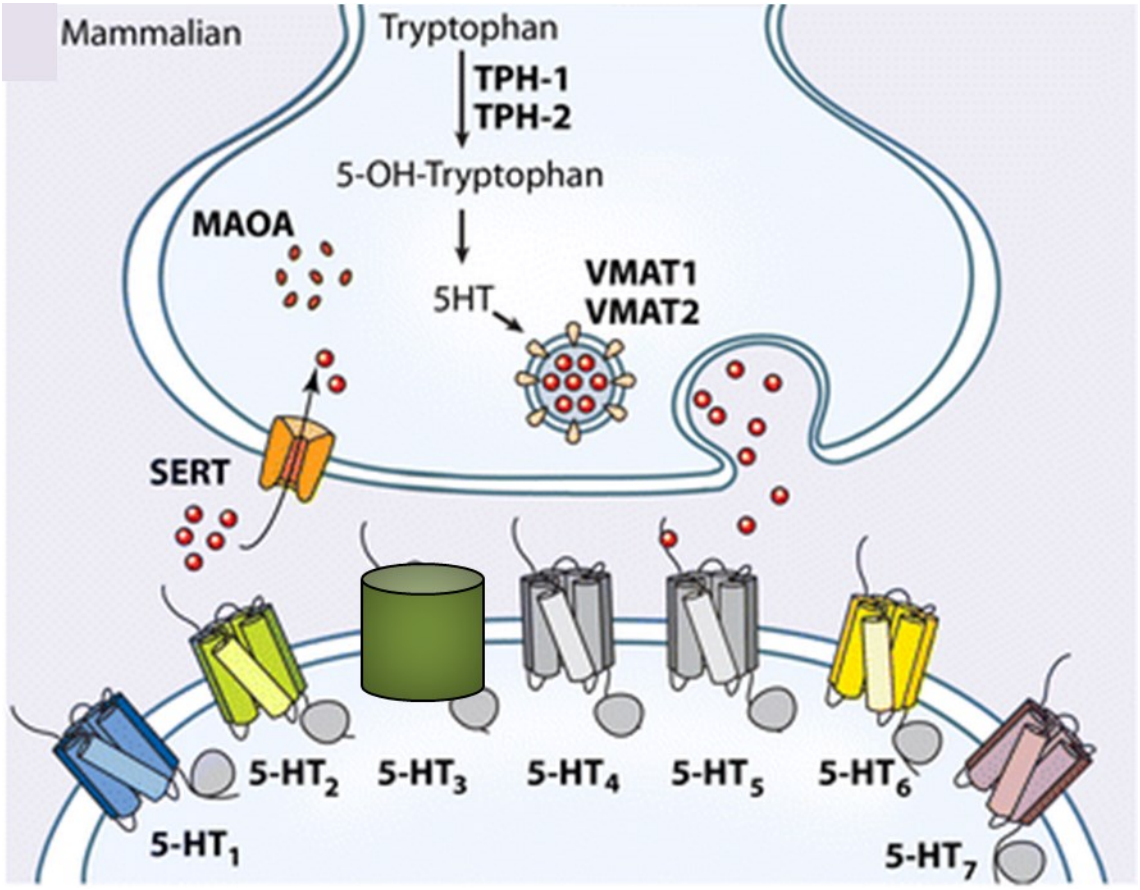


Figure 1.2 The mammalian serotonergic signaling (Curran and Chalasani 2012).

TPH: tryptophan hydroxylase. VMAT: Vesicular monoamine transporter.

MAO: monoamine oxidases. SERT: serotonin transporter. 5-HT_X: 5-HT receptors

Evolution of serotonin in the nervous system

Serotonin has a more ancient origin compared with the nervous system and first appeared in most unicellular organisms such as algae, where tryptophan, the amino acid from which serotonin is synthesized, is essential for converting solar energy to biological energy (Azmitia 2007). Serotonin is found to be secreted by an anaerobic unicellular organism gastrointestinal parasite *Entamoeba histolytica* and possibly functions as a signaling molecule for communication within the parasite population (Azmitia 2007, Dwyer 2007). It exerts a hormone-like effect to stimulate cilia regeneration in unicellular protozoan ciliate *Tetrahymena* (Castrodad, Renaud et al. 1988).

Serotonin was also found in plants and the concentration of serotonin in plant tissues is much higher than that in the animal nervous system (Azmitia 2007). Serotonin and melatonin regulate many cell differentiation processes in plants, such as mitosis, migration and maturation (Azmitia 2007).

As described above, serotonin was engaged in both intracellular and intercellular signaling long before the origin of nervous system or even the origin of synapse. When this signaling molecule became integrated as a component of the nervous system is not known. The concept of "protosynapse" points to the primitive organization of synaptic proteins in the most primitive form of animal life, as sponges, before the visible synapse (Ryan and Grant 2009). In sponges, serotonin-derived alkaloids were identified (Azmitia 2007). As an ancient molecule, the function of serotonin in the protosynapse remains to be addressed.

The most primitive nervous system was seen in *Cnidarians* and serotonin was localized in the sensory neurons of hydra, sea pansy and other members that belong to this phylum (Weiger

1997; Kass-Simon and Pierobon 2007). Serotonin's function in the earliest synapses in the phylum *Cnidaria* has not been much studied. It would be interesting to look at whether serotonin has been recruited as a neurotransmitter in all species of this phylum and also whether serotonin functions as a neurotransmitter or a neurohormone in these different species.

Overall, serotonin has an ancient origin and serotonergic neurons are present in all the phyla that possess a nervous system.

Anatomy of serotonergic system in different species

The localization and morphology of serotonergic neurons have been described in a variety of invertebrates and vertebrates. The vertebrates that have been studied include lamprey, the most primitive extant group of vertebrates (Abalo, Villar-Cheda et al. 2007; Barreiro-Iglesias, Villar-Cervino et al. 2008), garfish, (Parent and Northcutt 1982), goldfish (Bonn 1987), chicken (Dube and Parent 1981), rat (Lidov and Molliver 1982), monkeys ((Hornung and Fritschy 1988), and humans. In invertebrates such as molluscs, leeches and anthropods, serotonergic neurons are dispersed throughout the central nervous system among various ganglia (Gillette 2006), whereas in vertebrates, these neurons are concentrated in the raphe nuclei. However, in both cases, these serotonergic neurons innervate the CNS densely (Gillette 2006). These results not only provide evidences for the phylogenetic relation between these animals, but also indicate the increase of complexity of serotonergic system in vertebrates. For instance, the serotonergic system of lamprey resembles that of the jawed vertebrates but is different from that of most vertebrates (Abalo, Villar-Cheda et al. 2007; Barreiro-Iglesias, Villar-Cervino et al. 2008). The complexity of serotonergic systems in the brainstems of the avian lies intermediately between that of the

reptiles and the mammals (Dube and Parent 1981). These studies demonstrate that this basic component of the central nervous system is ancient and probably conserved.

Relatively fewer studies have compared the anatomy between species. One study compared the anatomy between 11 species of nudibranch mollusks, with both interspecies and intraspecies differences in the number of serotonergic neurons in each of the clusters (Newcomb, Fickbohm et al. 2006). Another study compared the serotonergic innervation of the motor cortex and the cortical area involved in working memory among macaques, chimpanzees and humans, aiming to identify a role of serotonin in human intellectual evolution. The authors identified a reorganization of cortical serotonergic transmission in humans and chimpanzees (Raghanti, Stimpson et al. 2008). This kind of anatomical comparison between closely related species would provide a foundation for comparative behavioral studies.

Serotonin regulates a broad range of animal behaviors in vertebrates and invertebrates

Serotonin in the nervous system modulates a broad range of animal behaviors including feeding, escape, sexual and aggressive behavior, learning and memory and in a more general sense, arousal (Weiger 1997). The cellular or molecular mechanisms underlying serotonin signaling have been well characterized in some of these behaviors. Serotonin exerts its effect as a classical neurotransmitter, a neuromodulator and a neurohormone in the level of sensory neurons, interneurons and motor neurons to regulate these behaviors.

Contraction of tissues

As initially discovered to "constrict" human intestine and blood vessels, serotonin in the nervous system also enhances contractions of multiple tissues across animal species. The case of *Cnidaria* is worth noticing, since it is the most primitive phylum with visible synapses and thus a

rudimentary nervous system (Ryan and Grant 2009). Sea pansy *Renilla koellikeri* is a species of this phylum. Serotonin-immunoreactive neurons were observed in the sea pancy and may be putative mechanosensory neurons based on morphology and localization (Tremblay, Henry et al. 2004). Exogenous serotonin increases the amplitude of spontaneous rhythmic contractions and induces the spawning of egg or sperm follicles in this organism (Tremblay, Henry et al. 2004). This behavioral modulation could be regarded as the most primitive role of serotonin in facilitating motor output.

Feeding behavior

The regulation of feeding behavior by nutrient state in invertebrates is significantly simpler than in mammals (Gillette 2006). In invertebrates, the nutritional status may be translated into appetitive states by the gut stretch signal, while in vertebrates, the central nervous system utilizes peptidergic hormones released from nutrient stores in the peripheral to monitor the nutrition status and regulate feeding behavior accordingly (Gillette 2006). Serotonin directly promotes appetitive state in invertebrates but inhibits feeding behavior in vertebrates (Gillette 2006).

Serotonin modulates of the feeding behavior in *Molluscs Aplysia californica*. In *aplysia*, a pair of large serotonergic neurons called MCCs (metacerebral cells) in the cerebral ganglion can be activated by the food stimuli. It is implicated that MCCs may positively modulate the activity of buccal motor neurons in buccal ganglion as well as the synaptic transmission from buccal ganglion motor neurons to buccal muscles. This modulation may increase the biting frequency and the biting speed, as evidenced by the observation that the biting will be weakened and slowed if MCC is hyperpolarized or selectively lesioned (Weiger 1997).

With a much more complicated nervous system, the leeches *Hirudo medicinalis, Macrobedella decora, Haementeria ghilianii* also recruit serotonin to promote their feeding

behavior (Weiger 1997). Three types of serotonergic neurons were identified in the leech: the Retzius (RZ) neurons, the large lateral (LL) neurons and the serotonergic interneurons. The feeding behavior consists of two phases: the swimming phase and the consummatory phase following it. In the swimming phase, mechanosensory stimuli, which could signal the presence of food, activates both the serotonergic interneurons and RZ neurons. The interneurons activate swim pattern generators through direct synaptic transmission and then receive a positive feedback from the swim pattern generators. RZ neurons, however, do not synaptically connect with the swim-related neurons. Instead, it releases serotonin as a neurohormone to promote swimming. In the consummatory phase, thermal stimulation and chemical stimulation from the food stimulate the lip of the animal, which in turn, activates RZ neurons and LL serotonergic neurons through synaptic connections. Activated RZ and LL neurons induce biting and salivary secretion during the consummatory phase. After feeding, body wall distension inhibits the activity of RZ and LL neurons and the serotonin level decreases in the animal (Weiger 1997).

Opposite to the case in invertebrates, results from pharmacological experiment support the notion that serotonin released from central nervous system exerts a predominant inhibitory effect on feeding in vertebrates (Gillette 2006, Donovan and Tecott 2013). For instance, acute inhibition of raphe nuclei neurons with GABA-A receptor agonist muscimol or application of TPH inhibitor p-chlorophenylalanine (PCPA) both caused hyperphagia and obesity, while injections of serotonin or 5-HTP resulted in hypophagia. Experiment results from genetic manipulations generated a complex result. Different TPH2 knockout lines resulted in inconsistent results. SERT null mice, instead of displaying hypophagia, exhibited adult-onset obesity instead. These discrepancies have been attributed to acute effects versus chronic effects and possible compensatory mechanisms (Donovan and Tecott 2013).

Vertebrate central serotonin is synthesized from the amino acid tryptophan, which is acquired from diet. Therefore, central serotonin level is highly correlated with tryptophan intake and serves as an indicator of short-term satiety (Donovan and Tecott 2013). In the vertebrate central nervous system, hypothalamus is the master regulator for behavioral states homeostasis, including feeding state. Two nuclei of the hypothalamus play essential roles for regulating appetite and energy balance. One of them is arcuate nucleus, located in the ventral medial aspect of hypothalamus. And the other group is orexin-releasing neurons, located in the lateral hypothalamus(Gillette 2006, Donovan and Tecott 2013). Central serotonin modulates the activity of both groups of neurons to suppress feeding behavior (Donovan and Tecott 2013). In the arcuate nucleus, there are two types of neurons regulating feeding behavior. The first group expresses the protein proopiomelanocortin (POMC), which is cleaved into several secreted peptides, including alpha melanocyte-stimulating hormone (a-MSH). The second group produces agouti-related peptide (AgRP) and neuropeptide Y (NPY). The POMC neurons inhibit feeding by activating melanocortin circuit downstream, while the AgRP/NPY neurons stimulate feeding by inhibiting POMC neurons and melanocortin circuit. Serotonin suppressed feeding by enhancing POMC neuronal activity through 5-HT2CR and inhibiting AgRP/NPY neuronal activity through 5-HT1BR (Donovan and Tecott 2013). In addition to modulating arcuate nucleus, serotonin also modulates orexin-releasing neurons through 5-HT1AR, although the mechanism is not as clear. Other than hypothalamus, central serotonin interacts with several other peptides that are essential for energy balance regulation, including leptin, ghrelin and insulin, and exerts modulatory effects through other serotonin receptors (Gillette 2006, Donovan and Tecott 2013).

Aggressive behavior

Serotonin has been strongly implicated to regulate aggressive behavior (Miczek, Fish et al. 2002).

It might be surprising to some people that male fruit fly *Drosophila melanogaster* can exhibit "aggressive behavior" as some much larger animals (Chen, Lee et al. 2002; Curran and Chalasani 2012). During fighting, two male fruit flies display wing threat, lunging, tussling and boxing against each other. Several different ways of enhancing serotonin signaling such as 5-HTP exposure or expression of dTrpA1 channels in serotonergic neurons resulted in escalated aggression and higher intense fighting, while inhibiting serotonin signaling disrupts the male fly's aggressive behavior (Alekseyenko, Lee et al. 2010; Curran and Chalasani 2012).

The modulation of aggressive behavior by serotonin has also been studied in east coast lobster *Homarus americanus* (Weiger 1997; Kravitz 2000) . When two lobsters encounter, they will engage in aggressive interactions. After fighting, one will assume a dominant posture and the other will have to assume a subordinate posture. Injection of serotonin into the haemolymph will produce the dominant posture, while the injection of octopamine will produce the subordinate posture (Weiger 1997). In terms of mechanisms, it seems that injection of serotonin alters central motor output to enhance postural flexion (Weiger 1997). A more recent analysis showed that serotonin reduces the likelihood of retreat and increases the duration of fighting (Huber, Smith et al. 1997).

Interestingly, the function of serotonin in modulating aggressive behavior in vertebrates seems to be opposite to that of invertebrates. Serotonin and norepinephrine (the analogue of octopamine in vertebrates) antagonize each other in regulating aggressive behavior in male

gymnotid fish (*Apteronotus leptorhynchus*), but the role appears to be opposite. Male fish use chirps, the increased electric organ discharge frequency, as an aggressive signal to other males. Intraventricular injection of serotonin decreases chirping while injection of norepinephrine increases chirping (Maler and Ellis 1987; Weiger 1997).

The relation between serotonin and aggression is studied extensively in rodents. Early analysis of serotonin receptors generated complex results (Haller 2013). But the general notion is that enhanced serotonin signaling suppresses aggression (Nelson and Chiavegatto 2001). This is supported by two recent studies. Tph2-deficient (Tph2(-/-)) mice exhibited strong aggressiveness as measured in the resident-intruder paradigm (Mosienko, Bert et al. 2012). And conditional overexpression of 5-HT1A in serotonergic neurons inhibited the neuronal activity of these neurons and increased aggression. 5-HT1A agonist application also increased aggression (Audero, Mlinar et al. 2013).

Learning behavior

The most famous study of serotonin's modulation of learning behavior is the sensitization of *Molluscs Aplysia californica* gill withdraw reflex studied by Nobel Laureate Eric Kandel (Kandel, Schwartz et al. 2000). Although this is only an implicit memory in invertebrates, the molecular and cellular mechanisms revealed by these studies are conserved even in more complex memory formation. The gill withdraw reflex is a defensive reflex displayed by the *Aplysia*. When the siphon of the sea slug receives a mild touch, both and siphon and the gill withdraw into the mantle cavity. This reflex can be sensitized by a noxious stimulus in the tail, for example, an electrical shock, so that the animal learns to respond more vigorously to the shock of the tail as well as the touch of the siphon. It is found that the tail sensory neurons activated by the shock excite serotonergic neurons called "facilitating interneurons", since

serotonin released from theses interneurons "facilitate" the activation of the siphon sensory neurons that activates the gill motor neurons (Kandel, Schwartz et al. 2000). The molecular mechanism for this facilitation is very well characterized that serotonin binds with serotonin receptors on the siphon sensory neuron membranes and activates downstream signaling pathways including PKA in the short term sensitization; and PKA and CREB transcription factors in the long term sensitization (Kandel, Schwartz et al. 2000).

Another species of *Molluscs***,** the sea slug *Tritonia diomedea*, has a similar defensive reflex as the gill withdraw in *Aplysia californica*, the escape swimming reflex (Brown 1998). Serotonin is released from interneurons and may also play a modulatory role in sensitization of this reflex. Three dorsal swimming interneurons (DSI) are serotonin-immunoreactive. They activate downstream neurons through direct synaptic connections and also modulate the connections between other neurons in a heterosynaptic manner. This modulation role of these serotonergic interneurons may sensitize that escape swimming behavior because after animals are exposed to a swim-motivating stimulus, they have a decreased latency to start swimming in response to further stimulation (Weiger 1997; Brown 1998; Frost, Brandon et al. 1998).

Comparison of one particular behavior among closely related species may reveal the evolution of the behavior and the neuronal circuit underlying it. In one example of this type of studies, the researchers compared the sensitization of gill withdraws among three *Molluscs* species *Aplysia californica*, *Phyllaplysia taylori* and *Dolabrifera dolabrifera* (Marinesco, Duran et al. 2003). Although the anatomy of serotonergic system is similar among three species, the animal behaviors displayed interspecific variations in a modified sensitization paradigm. Instead of touching the siphon and shocking the tail, the researchers shocked one side of the tail and touched the same side or the other side of the tail. If both the shock and the touch were on the

same side of the tail, it was called "local sensitization". If the sites were different, it was called "general sensitization". *Aplysia* displayed both forms of sensitization while *Phyllaplysia* only showed the local sensitization and *Dolabrifera* had no sensitization at all. To explain the observed behavioral differences, the authors examined the neurobiological correlate of sensitization, which is the heterosynaptic modulation of sensory neuron excitability by tail shock. Although serotonin release showed interspecies differences, it did not correlate with the behavior at all. The post-synaptic response to serotonin in the tail sensory neurons seemed to be highly correlated with the behavioral differences. *Aplysia* and *Phyllaplysia's* ipsilateral tail sensory neurons' excitability was increased while the sensory neurons' excitability of *Dolabrifera* was not changed after the shock. The result of this study suggested that the sensitization of *Aplysia* may be relatively recent and the postsynaptic machinery responding to serotonin evolved after the recruitment of serotonin as a neurotransmitter. (Marinesco, Duran et al. 2003).

In a similar example of this type of study, the authors compared the escape swimming behavior in another three closely related species *Tritonia diomedea*, *Pleurobranchaea californica* and *Hermissenda crassicornis*. Although the neuronal circuit is conserved, *Hermissenda crassicornis* did not exhibit an analogous swimming behavior. Correspondingly, in *Tritonia* and *Pleurobranchaea*, but not in *Hermissenda*, the serotonergic DSI homologs modulated the strength of synapses made by downstream neurons (Lillvis and Katz 2013).

Serotonin also regulates learning behavior in insects. One example is the place memory in fruit fly *Drosophila melanogaster*. By blocking the neurotransmission of serotonergic neurons specifically in the brain of fruit fly with Gal-4/Gal-80 driven TeTx or pharmacological manipulations with α-methyl tryptophan (am-W) to inhibit the synthesis of serotonin, it was

shown that serotonin is necessary for the place memory negatively reinforced by high-temperature in the fruit fly (Sitaraman, Zars et al. 2008).

Another example of serotonin regulating learning in insects is the food conditioned olfactory aversive learning in honeybee *Apis mellifera* (Wright 2011). The aversive behavior of honeybee can be measured through the proboscis extension reflex and there are two forms of olfactory aversive learning. The first form is pre-ingestive: the honeybee detects the toxin through gustatory sensation and learns to avoid the odor associated with the toxic food. The second form of learning is post-ingestive: the honeybee remembers to avoid the odor associated with the toxic food, which after ingestion causes malaise. The first form of learning is dependent on dopamine while the second form of learning is dependent on serotonin, as demonstrated by blocking dopamine or serotonin receptors (Wright 2011). It is interesting to notice that both the neurotransmitter and the behavior for the second form of olfactory aversive learning are similar in both honey bee and *C. elegans* (Zhang, Lu et al. 2005), which will be discussed later.

Finally, the study of serotonin mediating learning in rodents generated mixed results, depending on the context and the type of learning. In a central serotonin-deficient mice line, the retrieval of spatial memory tested in the hidden platform assay was impaired while the context fear memory generated by foot shock was enhanced. Surprisingly, this was not due to the timidity of these central serotonin deficient mice, since their anxiety-like behavior was reduced as tested in the elevated-plus maze and novelty-suppressed feeding test (Dai, Han et al. 2008).

Serotonin dysregulation and its implication in human mood disorders

Serotonin modulates human emotion states (stress, fear and aggression), appetite, sleep and so on. Since the monoamine hypothesis of depression was proposed, the association between serotonergic signaling and human mood disorders has received substantial attention.

The monoamine hypothesis of depression originally came from several observations in the antidepressant drug development history. During the first half of the 20th century (before 1950s), electroconvulsive therapy was the treatment of choice for depressed patients (Lieberman 2003). In 1950, it was reported that reserpine, a Rauwolfia alkaloid used for the treatment of hypertension, produced depression-like syndrome by depleting the brain serotonin and norepinephrine (Kandel, Schwartz et al. 2000). Following this observation, monoamine oxidase inhibitors (iproniazid), which were developed to treat tuberculosis, were found to elevate mood in depression patients in clinical studies (Zeller, Barsky et al. 1955; Crane 1956). Meanwhile, tricyclic antidepressant imipramine (Tofranil® by Ciba, nowadays Novartis) was also developed, although it was first tried against psychotic disorders (such as schizophrenia) (Kuhn 1957). The monoamine hypothesis of depression was formed during this era. The serotonin hypothesis and the catecholamine hypothesis were of wide interests and actively discussed (Coppen 1967). In 1970s, fueled by the serotonin hypothesis of depression, the selective serotonin reuptake inhibitor fluoxetine (SSRI, Prozac® developed by Eli Lilly) was developed as the first-line antidepressant (Fuller, Perry et al. 1974), supporting a role of enhanced serotonin transmission in the treatment of depression.

As a target of antidepressant drugs, the polymorphisms of serotonin transporter (SERT) were examined in a number of human genetics studies. SLC6A4 is the human gene encoding SERT. The promoter region of SLC6A4 contains a common length repeat polymorphism: *l* form with

16-repeat units and *s* form with 14-repeat units. With the candidate gene approach, the initial study showed that the *s* form had reduced transcriptional activity in human lymphoblastoid cell lines and was significantly associated with anxiety-related traits and depression-related sub-factors, although the inherited variance explained by the SERT polymorphisms was quite small (Lesch, Bengel et al. 1996). Follow-up studies showed inconsistent results (Canli and Lesch 2007), although the most recent meta-analysis repeated the association between *s* allele and depression (Clarke, Flint et al. 2010). Interestingly, SSRI and the *s* allele both decreased 5-HT reuptake, but showed different effects in the depression disorder. The author explained that the effect of reduced SERT function could be different in patients than in general population. Also the SLC6A4 polymorphisms had a chronic effect since early development and this is different from the effect of SSRI (Lesch, Bengel et al. 1996).

How does serotonin regulate stress response? Serotonin interacts with the hormonal hypothalamic-pituitary-adrenal (HPA) axis and amygdala to modulate the stress response (Chen and Miller 2012; Curran and Chalasani 2012). As a homeostasis master regulator, one of the functions of the hypothalamus is to regulate the stress response. The stress response prepares the organism to adapt to the changed environment and re-establish the homeostasis. Stress stimulus activates parvocellular neurosecretory neurons in the paraventricular nucleus (PVN) of hypothalamus to release corticotropin-releasing hormone (CRH) to anterior pituitary. Anterior pituitary will be triggered by CRH to release adrenocortocotropic hormone (ACTH), which goes into the circulation to the peripheral to trigger the release of glucocorticoid (cortisol in primates and corticosterone in rodents) from the adrenal cortex (Bear, Connors et al. 2007). Glucocorticoid can change the expression of an array of genes and exert an extensive effect on metabolism, cardiovascular function, the inflammatory/immune response, cognition, and so on

(Chen and Miller 2012). Meanwhile, elevated glucocorticoid level suppresses the release of CRH and ACTH level through a negative feedback (Chen and Miller 2012) (Figure 1.3). Besides regulating neurohormone level, hypothalamus also re-establishes the homeostasis by regulating the motor response and autonomic response through activation of sympathetic nervous system (Bear, Connors et al. 2007).

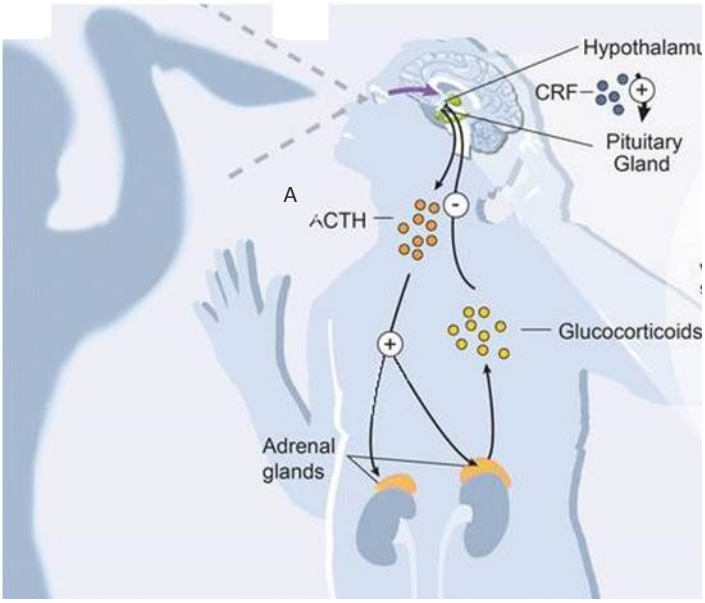


Figure 1.3 Physiological processing of stress stimulus through the hypothalamic-pituitary-adrenal (HPA) axis (Curran and Chalasani 2012).

CRF or CRH: corticotrophin-releasing factor/hormone. ACTH: adrenocortocotropic hormone. Stress stimulus activates hypothalamus to release corticotropin-releasing hormone (CRH) to anterior pituitary, which releases adrenocortocotropic hormone (ACTH). ACTH goes into the circulation to the peripheral to trigger the release of glucocorticoid from the adrenal cortex. Elevated glucocorticoid suppresses the release of CRH and ACTH.

On the other hand, the amygdala is a cluster of nuclei activated by stress/fear stimulus, as is identified by functional imaging (Curran and Chalasani 2012). When amygdala is activated by stress stimulus through sensory system, amygdala in turn activates HPA axis (Bear, Connors et al. 2007).

Serotonergic neurons project to PVN of hypothalamus and amygdala (Curran and Chalasani 2012). CRH and glucocorticoid also modulate the synthesis/turnover and release of serotonin from the raphe nuclei. Mice study shows that repeated administration of the dexamethasone (the synthetic glucocorticoid) induced a significant decrease of TPH2 level (Clark, Pai et al. 2005; Clark, Flick et al. 2008). However, studies in rat showed the opposite conclusion that glucocorticoid induced tph2 mRNA expression and serotonin signaling (Clark, Flick et al. 2008; Barr and Forster 2011). These results implicated the complexity in the interaction between serotonergic system and the HPA axis.

Post mortem analysis of human brain tissues also shed light on the regulation of serotonin signaling by the stressful events. One of the studies showed the number and density of dorsal raphe nuclei serotonergic neurons were elevated in suicide victims (Underwood, Khaibulina et al. 1999). A study from the same research group later reported that TPH immunoreactivity in dorsal raphe and median raphe is higher in depressed suicides (Boldrini, Underwood et al. 2005) and tph2 mRNA level is higher per neuron in depressed suicides (Bach-Mizrachi, Underwood et al. 2006; Bach-Mizrachi, Underwood et al. 2008). However, studies from another research group showed a different result. The TPH2 protein level did not increase in the dorsal raphe nuclei of depressed suicide victims, but increased in the dorsal subnucleus of dorsal raphe in the alcohol-dependent depressed suicides (Bonkale, Murdock et al. 2004; Bonkale, Turecki et al. 2006). Consistent with these reports, tph2 transcripts levels in ventral prefrontal cortex, where

serotonergic neurons projected to, were also higher in suicides (Perroud, Neidhart et al. 2010). This increase of TPH2 level might be a compensation response to the decreased 5-HT level as measured from the low cerebrospinal fluid level of 5-HIAA in depressed patients with well-planned suicide attempts (Mann and Malone 1997).

Human genetics research has moved from the candidate approach and linkage analysis to genome-wide association study. However, compared with other complex human disorders, not much new information is gained from genome-wide association study for major depression. No SNP reached genome-wide significance in the most recent mega-analysis of genome-wide association studies for major depressive disorder, with 9240 cases and 9519 controls (Ripke, Wray et al. 2013).

Obviously, the etiology underlying depression is much more complicated than dysregulation of serotonin signaling only. Besides the results from human genetics study mentioned above, it is worth noticing that it usually takes 4-6 weeks for SSRI to achieve clinical antidepressant efficacy (Lutz 2013) and only one-third of patients exhibit a beneficial therapeutic response after treated with antidepressant drugs (Narasimhan and Lohoff 2012). However, test carried in rats to compare all antidepressant strategies showed a striking commonality that 5-HT transmission overall is enhanced by these treatments, including electroconvulsive shock and relatively novel treatment strategies such as sleep deprivation and deep brain stimulation (Blier and El Mansari 2013). Although it is possible that enhancement of serotonergic system have therapeutic effects on depression without a etiological cause, these observations still argue for the importance to examine the effect on serotonergic signaling by stress stimulus per se.

Aversive experience enhances serotonergic neurons activity and increases tph2 mRNA level in rodents

Studies in rodents showed that different types of aversive experience enhance serotonergic neurons activity in different parts of raphe nuclei when the neuronal activity was measured by c-Fos expression in these neurons (Gaspar and Lillesaar 2012). In rats, social defeat increased the number of c-Fos-like immunoreactive serotonergic nuclei in dorsal part of dorsal raphe (Gardner, Thrivikraman et al. 2005; Gaspar and Lillesaar 2012), fear conditioning increased c-Fos expression throughout dorsal raphe and fear potentiated startle increased dorsal part of dorsal raphe (Spannuth, Hale et al. 2011; Gaspar and Lillesaar 2012).

On the other hand, stressful events also regulate TPH level as shown in rodent studies. In the rat raphe nuclei, TPH2 mRNA level is elevated by stress stimuli, such as repeated immobilization stress (Chamas, Serova et al. 1999; Chamas, Underwood et al. 2004), repeated forced swimming (the same stimulus deceased 5-HT level and 5-HIAA/5-HT ratio in hypothalamus and amygdala) (Shishkina, Kalinina et al. 2008), adverse early life experiences and interaction with adulthood social defeat (Gardner, Hale et al. 2009). TPH2 mRNA level is also increased in both dorsal and medial raphe in male mice suffering from chronic variable stress (McEuen, Beck et al. 2008).

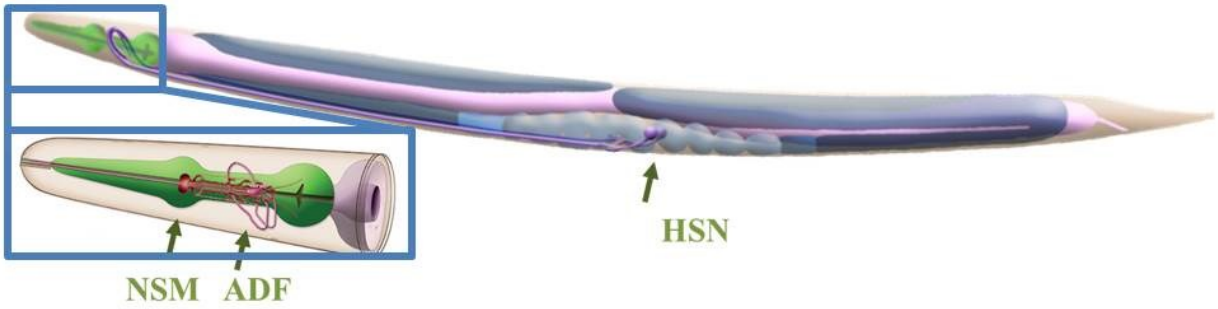
Serotonin regulates multiple behaviors in *Caenorhabditis elegans*

In *Caenorhabditis elegans*, several neurons showed serotonin immunoreactivity: a pair of NSM neurosecretory-motor neurons, a pair of ADF chemosensory neurons, a pair of HSN motor neurons, a pair of AIMs and a RIH neuron (Kullyev, Dempsey et al. 2010). The *C. elegans* tryptophan hydroxylase, TPH-1, is highly homologous to the mammalian TPH and is essential

for serotonin biosynthesis. An expression reporter of *C. elegans tph-1* is expressed in three pairs of serotonin-producing neurons, NSM, ADF, and HSN (Sze, Victor et al. 2000). On top of the serotonin producing neurons, AIM, RIH neurons express serotonin reuptake transporter *mod-5* and are able to re-uptake serotonin from the synaptic cleft and release it as the neurotransmitter (Figure 1.4 B) (Jafari, Xie et al. 2011). NSM and ADF neurons also express *mod-5*, while HSN neurons do not express *mod-5* and cannot uptake extrasynaptic serotonin (Jafari, Xie et al. 2011). Although it was reported VC4/5 neurons exhibit weak and variable serotonin immunoreactivity (Duerr, Frisby et al. 1999), it was not clear if these neurons express *mod-5* reuptake transporter (Jafari, Xie et al. 2011).

At least five types of serotonin receptors have been found to be expressed in *C. elegans* neurons. Four of these serotonin receptors (SER-1, SER-4, SER-5, SER-7) have mammalian homologs, while one of them MOD-1 is a 5-HT gated chloride channel without a homolog in mammals (Curran and Chalasani 2012).

A



B

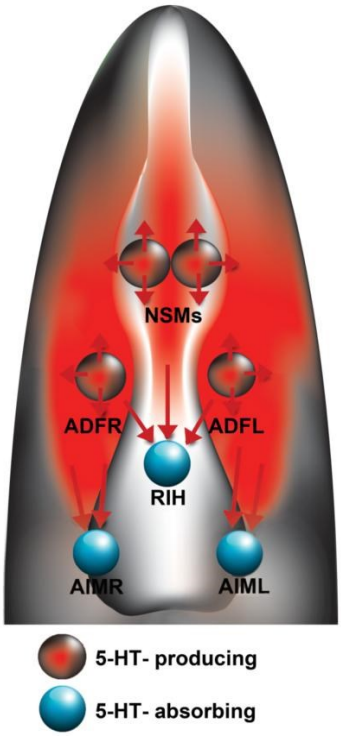


Figure 1.4 The serotonergic system of *C. elegans.*

A, The position of 5-HT producing neurons on the body of *C. elegans* (figure produced from wormatlas figures).

B, 5-HT-producing and 5-HT-absorbing neurons in *C. elegans* (Jafari, Xie et al. 2011).

The effort to characterize serotonin in regulating *C. elegans* behavior started with pharmacological approaches, as in other organisms. The pharmacological behavioral analysis with exogenous applied serotonin and serotonin related drugs were followed by genetic analysis of serotonin mutants.

Exogenous serotonin inhibits locomotion and stimulates pharyngeal pumping and egg-laying in *C. elegans* (Horvitz, Chalfie et al. 1982). The effect of exogenous serotonin and low concentration of serotonin uptake inhibitor imipramine on the pharyngeal pumping behavior mimics the effect of the food (Avery and Horvitz 1990). On the other hand, the *tph-1(mg280)* null mutant animals pump slower on bacteria lawn, retain more eggs (Sze, Victor et al. 2000), and move faster in the presence of food (Cunningham, Hua et al. 2012) compared with wild type animals. In addition, 10-15% of the mutants arrest at the dauer state even in presence of food (Sze, Victor et al. 2000). The phenotypes of *tph-1(mg280)* mutant are similar to the phenotypes of starved worms (Avery and Horvitz 1990; Weinshenker, Garriga et al. 1995). Thus endogenous serotonin also signals the presence of food. Another behavior modulated by serotonin is the avoidance response to diluted octanol in *C. elegans*. Both food presence and exogenous serotonin enhance the avoidance against diluted octanol (Chao, Komatsu et al. 2004; Harris, Hapiak et al. 2009). Similar to the series of phenotypes mentioned above, *tph-1(mg280)* mutant displayed a weak avoidance against octanol even on food (Chao, Komatsu et al. 2004).

Neuronal ablation experiments and cell specific rescue of *tph-1* mutant better defined the function of serotonin from specific neurons. Analysis of serotonergic neurons and neurons expressing serotonin receptors expanded the research to the study of serotonergic circuits underlying behaviors.

As mentioned earlier, HSN neurons are the hermaphrodite specific motor neurons located lateral and slightly posterior to the vulva. On top of serotonin, HSNs also utilize acetylcholine (Duerr, Gaskin et al. 2001), neuropeptide-like protein NLP-3, NLP-8, NLP-15 (Nathoo, Moeller et al. 2001) and FMRFamide-like neuropeptide FLP-5 (Kim and Li 2004). The function of HSNs has been studied extensively in the egg-laying behavior. The egg-laying system of *C. elegans* consists of two classes of motor neurons, VCs (hermaphrodite-specific ventral nerve cord neurons) and HSNs, both innervating vulval muscles (White, Southgate et al. 1986; Desai and Horvitz 1989). HSNs make synaptic connections with VC5 motor neuron (White, Southgate et al. 1986). VC5 utilizes Ach as its neurotransmitter (Duerr, Gaskin et al. 2001). In addition, VC5 is stained weakly for serotonin (Duerr, Frisby et al. 1999) and express vesicular monoamine transporter (Duerr, Frisby et al. 1999; Duerr, Gaskin et al. 2001).

Serotonin from HSNs is shown to induce the active state of egg-laying based on two pieces of evidence: First, exogenous serotonin rescues egg-laying defects in several egg-laying defective mutants (Trent, Tsuing et al. 1983); Second, HSN-ablated animals lay eggs in a slower rate mainly due to the prolonged inactive state, while once entering the active state, they lay eggs just as wild type animals (Waggoner, Zhou et al. 1998). It is worth noticing that *tph-1* mutant is not as defective as HSN ablated animals in egg-laying (Weinshenker, Garriga et al. 1995; Kim, Poole et al. 2001). One of the possibility is that serotonin from HSN neurons plays both excitatory and inhibitory roles. Analysis of combinations of serotonin receptors showed that indeed serotonin exhibits both positive and negative role for egg-laying through different receptors (Carnell, Illi et al. 2005; Hobson, Hapiak et al. 2006; Hapiak, Hobson et al. 2009). Consistently, calcium imaging analysis showed that serotonin increases calcium frequency in vulval muscles and VC neurons, but inhibits HSN spontaneous activity (Shyn, Kerr et al. 2003;

Zhang, Chung et al. 2008). The other possibility to explain the weaker phenotype of *tph-1* mutant is that other neurotransmitters from HSN is also promoting egg-laying. HSN activates egg-laying event by activating vulval muscles directly and indirectly through VC neurons (Zhang, Chung et al. 2008). When *cha-1(y226)* mutant, which is defective in the choline acetyltransferase that synthesizes acetylcholine, is transferred to restrictive temperature, VC calcium activity is inhibited, suggesting that acetylcholine from HSN neurons is activating VC neurons for the egg-laying behavior (Zhang, Chung et al. 2008).

NSM neurons are a pair of neurosecretory-motor neurons near the pharynx that synthesize and release serotonin (Horvitz, Chalfie et al. 1982; Sze, Victor et al. 2000), glutamate (Lee, Sawin et al. 1999), and also release NLP-13, NLP-18 and NLP-19 (Nathoo, Moeller et al. 2001). NSM neurons have sensory endings in the pharynx, the outside of pharynx and the pseudocoelom (Chase 2007), which appears to be a form of the circulation system of the animal, thus serotonin released from this pair of neurons can function as a neurotransmitter as well as a neurohormone. Serotonin from NSM neurons regulates food signal regulated locomotion (Sawin, Ranganathan et al. 2000) and food signal regulated aversive response to diluted octanol (Harris, Korchnak et al. 2011).

Serotonin regulates the locomotion of *C. elegans* in response to its feeding state and feeding environment. While well-fed animals move more slowly when encounter bacteria, starved animals reduce their moving speed even more dramatically when they are on a bacteria lawn. Serotonin is required for this "enhanced slowing response" and worms with NSM neurons ablated show a small but significant defect in this behavior (Sawin, Ranganathan et al. 2000). Expression of *tph-1* cDNA in NSM neurons but not in ADF neurons of *tph-1* mutant is sufficient to rescue the enhanced slowing response (Zhang, Lu et al. 2005). Both pieces of evidence

suggest that serotonin from NSM neurons promote the enhanced slowing response in starved animals. Interestingly, pharmacological analysis showed that fluoxetine blocks serotonin uptake and when applied at low concentration, fluoxetine potentiate enhanced slowing response through inhibition of *mod-5* (Ranganathan, Sawin et al. 2001). This effect could be due to depression of auto-inhibition of NSM neurons or up-regulate synaptic serotonin in general.

There is evidence to show that both exogenous serotonin and endogenous serotonin enhance animals' aversive response to dilute (30%) octanol (Chao, Komatsu et al. 2004; Harris, Hapiak et al. 2009). *tph-1(mg280)* mutant is defective in this food enhanced avoidance (Chao, Komatsu et al. 2004; Harris, Korchnak et al. 2011) and this defect can be rescued by expression of *tph-1* cDNA in NSM neurons (Harris, Korchnak et al. 2011). Consistently, NSM cell specific knockdown of *tph-1* in the wild type background generated a *tph-1(mg280)* mutant-like phenotype (Harris, Korchnak et al. 2011). Interestingly, serotonin from ADF neurons seemed to play an opposite role in regulating this behavior. While NSM serotonin mimics a food-like signal, ADF serotonin signals to antagonize the stimulatory role of the food for this aversion response (Harris, Korchnak et al. 2011).

In addition to these behaviors, serotonin from both NSM and HSN neurons can extend dwelling state during forage (Bargmann, 2012 Cell symposia).

ADF neurons was first classified as a chemosensory neuron due to its ciliated endings exposed to the environment (Bargmann and Horvitz 1991). Besides serotonin, ADF neurons may release FLP-6, INS-1 and NLP-3 (Li and Kim 2008). From laser ablation experiments, it was shown that ADF neurons together with ASG and ASI neurons were responsible for the small residual chemotactic response to cAMP, biotin, Cl-, and Na+, after ASEs are killed (Bargmann

and Horvitz 1991). It was also shown that laser ablation of ADF, ASI, ASJ and ASG neurons caused a high percentage of worms go into dauer, and ADF neurons together with ASI and ASG neurons inhibited entry into dauer stage (Bargmann and Horvitz 1991). However, it was not until very recently that the specific function of serotonin from ADF neurons was identified.

The serotonin from ADF neurons was shown to be required for the memory formation in the olfactory aversive learning paradigm. In this learning paradigm, aversive training of *C. elegans* with pathogenic bacteria, such as *P. aeruginosa* PA14, induces olfactory aversion in the worms to the smell of the pathogenic bacteria (Zhang, Lu et al. 2005). Naive worms that are never exposed to the pathogenic bacteria PA14 often prefer the smell of PA14, whereas trained worms that have been infected by the pathogenic bacteria avoid the bacterial smell. This form of behavioral plasticity requires the activity of the *C. elegans* TPH-1 in ADF serotonergic neurons. Because *tph-1* null mutant worms, which cannot produce serotonin, are defective in this olfactory aversive learning behavior and selective expression of *tph-1* cDNA in the ADF neurons of the *tph-1* null mutants restores the learning defect (Zhang, Lu et al. 2005).

Another function of serotonin from ADF neurons is promoting hyperoxia avoidance (Chang, Chronis et al. 2006). *tph-1* mutant worms avoided hyperoxia about half of the wild type level and expression of *tph-1* cDNA in ADF neurons rescued the hyperoxia avoidance defect (Chang, Chronis et al. 2006). Interestingly, as locomotion and feeding behaviors mentioned above, hyperoxia avoidance behavior can also be regulated by the presence of food. The presence of food strongly suppressed the hyperoxia avoidance in wild type background, but the half wild type hyperoxia level in *tph-1* mutant cannot be suppressed by food (Chang, Chronis et al. 2006). The ADF specific expression of *tph-1* cannot rescue this food modulation of hyperoxia. The

authors explained that either endogenous *tph-1* promoter or other serotonergic neurons are required to show the effect of food (Chang, Chronis et al. 2006).

Exogenous serotonin stimulates pharyngeal pumping (Horvitz, Chalfie et al. 1982). Food signal also stimulates pharyngeal pumping (Avery and Horvitz 1990). *tph-1(mg280)* mutant on average pump slower in the presence of food than wild type (Hobson, Hapiak et al. 2006; Song and Avery 2012). This suggests that endogenous serotonin also stimulates pumping in present of food. One of the early studies showed that NSM ablated animals pump as wild type in the presence of food (Avery and Horvitz 1989). Consistent with the conclusion from the early study, reconstitution of *tph-1* cDNA in ADF neurons, not NSM neurons, restored the pumping rate of *tph-1* mutant on food to the level of the wild type (Cunningham, Hua et al. 2012). Moreover, knockdown of *tph-1* expression in ADF neurons can decrease the pumping rate of wild type animals to the level of *tph-1* mutant (Cunningham, Hua et al. 2012). Interestingly, feeding behavior seems to be regulated not only by food, but also by familiar food (Song, Faumont et al. 2013). The pumping rate of wild type animals on familiar food is higher than the pumping rate on novel bacteria (Song, Faumont et al. 2013). But this regulation is absent in *tph-1* mutant, and again, this mutant defect can be restored by expression of *tph-1* in ADF neurons (Song, Faumont et al. 2013). All these data suggest that similar to NSM neurons, ADF neurons can also be modulated by the food signal or familiarity/novelty of food. In both cases, the downstream targets of ADF neurons – AVJ neurons for food enhanced pharyngeal pumping and MC neurons in pharynx for familiar food enhanced pumping – do not have direct synaptic connection with ADF neurons. Both studies showed that the function of *mod-5* is not required for ADF serotonin's effect (Cunningham, Hua et al. 2012; Song, Faumont et al. 2013), suggesting that ADF serotonin can act as an endocrine signal. Besides food signal, attractive odor such as

diacetyl can also stimulate pharyngeal pumping (Li, Li et al. 2012). Similar to the case of food stimulating pumping, *tph-1(mg280)* mutant is defective in pumping stimulated by diacetyl (Li, Li et al. 2012). However, different from the two studies mentioned above, expression of *tph-1* cDNA specifically in NSM neurons not in ADF neurons rescues the mutant phenotype in this behavior (Li, Li et al. 2012). It is not clear whether the downstream target of NSM serotonin in this diacetyl enhanced pumping behavior is similar to the target of ADF serotonin in the food signal enhanced pumping behavior.

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Chapter 2

Cellular and molecular mechanisms underlying *tph-1* transcription up-regulation in *C. elegans* aversive olfactory learning

Introduction

Aversive experience regulates *tph-1* levels in the *C. elegans* serotonergic neurons ADF

The transcription of *C. elegans tph-1* in ADF serotonergic neurons is dynamically regulated by various conditions. Heat stress increased *tph-1* level in ADF neurons (Estevez, Estevez et al. 2004; Moussaif and Sze 2009). *tph-1* level is higher in dauers, which is a special developmental stage induced by long-term starvation in the larvae (Estevez, Cowie et al. 2006; Moussaif and Sze 2009; Xie, Moussaif et al. 2013). However, 4 hours starvation in adults decreased the *tph-1* level in ADF neurons (Liang, Moussaif et al. 2006; Cunningham, Hua et al. 2012). All these data are consistent with the modulatory role of serotonin in behaviors that are affected by these conditions (Chase and Koelle 2007).

The pathogenesis model of *C. elegans* infection by *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is a gram-negative bacterium found in soil and aquatic environments (Tan, Mahajan-Miklos et al. 1999). It was shown to be pathogenic to plants, *C. elegans*, insects, and a variety of vertebrates including mice and human (Tan, Mahajan-Miklos et al. 1999). Several *P. aeruginosa* strains can kill *C. elegans* (Tan, Mahajan-Miklos et al. 1999). Among these *P. aeruginosa* strains, *P. aeruginosa* PA14, which is a single clinical isolate of the human opportunistic pathogen *P. aeruginosa*, is the most lethal (Tan, Mahajan-Miklos et al. 1999). The killing of *C. elegans* by PA14 is associated with establishment and proliferation of PA14 within the worm's intestine and is an active infection-like process (Tan, Mahajan-Miklos et al. 1999). By investigating the interaction between *C. elegans* and PA14, a pathogenesis model was established to study the pathogen virulence factors and host defense mechanisms. For instance, PA14 *gacA*, which will be discussed further in Chapter 3, is a virulence factor identified by using this pathogenesis model (Tan, Rahme et al. 1999). Also, the NSY-1-SEK-1-PMK-1 pathway,

which is orthologous to mammalian ASK1 MAPKKK-MKK3/6 MAPKK-p38 MAPK pathway (Kim, Feinbaum et al. 2002), was found to function downstream of the homologue of human SARM, Toll-Interleukin-1 Receptor /Resistance (TIR) domain protein TIR-1, and act in the intestine cell autonomously to regulate innate immunity in *C. elegans* (Couillault, Pujol et al. 2004; Liberati, Fitzgerald et al. 2004).

The pathogenic bacteria *Pseudomonas aeruginosa* induces olfactory aversive learning and serotonin (*tph-1*) up-regulation in the *C. elegans* serotonergic neurons ADF

As mentioned in Chapter 1, as a bacteria-feeding nematode, *C. elegans* learns to avoid the smell of pathogenic bacteria after ingestion (Figure 2.1 A, B). This learning process requires the serotonergic signal from ADF neurons. Intriguingly, aversive training with pathogenic bacteria increases *tph-1* transcription (Figure 2.1 C) and the serotonin content in ADF, and the ADF serotonin signal promotes learning through several interneurons (Zhang, Lu et al. 2005). These findings further demonstrated the importance of *tph-1* regulation and provided a system to examine its underlying mechanisms. One of the previous studies reported the *C. elegans* innate immunity regulator TIR-1-NSY-1-SEK-1 pathway, but not PMK-1, is essential for the upregulation of ADF *tph-1* by aversive training (Shivers, Kooistra et al. 2009). Dr. Xiaodong Zhang and I observed a normal upregulation in *nsy-1(ky397)* (data not shown). This inconsistency could be due to different training conditions or different allele effects. In this chapter, I describe the characterization of the mechanisms underlying *tph-1* transcriptional upregulation induced by aversive experience with PA14 infection, from the level of neuronal physiology, molecular genetics and circuit analysis.

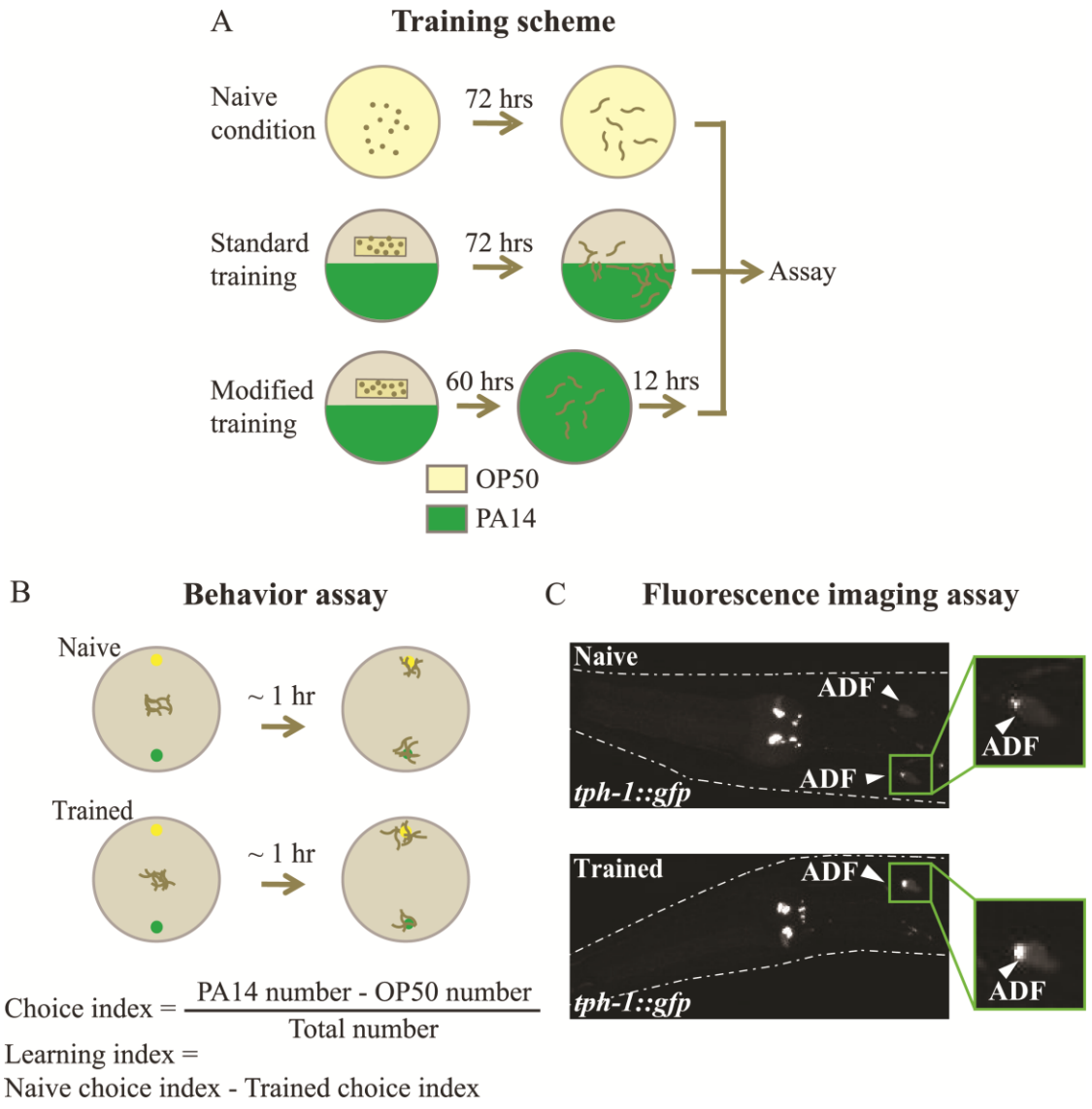


Figure 2.1 Long-term training procedure, aversive olfactory learning assay and fluorescence imaging assay for *tph-1* transcription quantification.

A, Standard and modified training procedure with *P. aeruginosa* PA14. ***B,*** Schematic for the aversive olfactory learning assay. ***C,*** Images of *tph-1*::*gfp* expression in naive or trained animals. Arrowheads indicate ADF; dashed lines outline the worms.

Results

Long-term aversive training enhances the sensory-evoked calcium response of the ADF neurons

To understand how *tph-1* transcription in ADF neurons is regulated by training experience, I first examined the ADF neuronal physiology by performing intracellular calcium imaging since ADF neuronal activity has not been studied before our study. To fulfill this purpose, I built the calcium imaging system for the lab according to what is established before (Chronis, Zimmer et al. 2007). A genetically encoded calcium sensor, Cameleon YC3.60 (Nagai, Yamada et al. 2004), was expressed in ADF using the ADF specific *srh-142* promoter and recorded the calcium response of transgenic animals in a microfluidic behavior chip (Figure 2.2 A, B) (Chronis, Zimmer et al. 2007). When Cameleon YC3.60 is expressed in neurons, the increase of YFP-to-CFP ratio indicates the elevation of calcium transients.

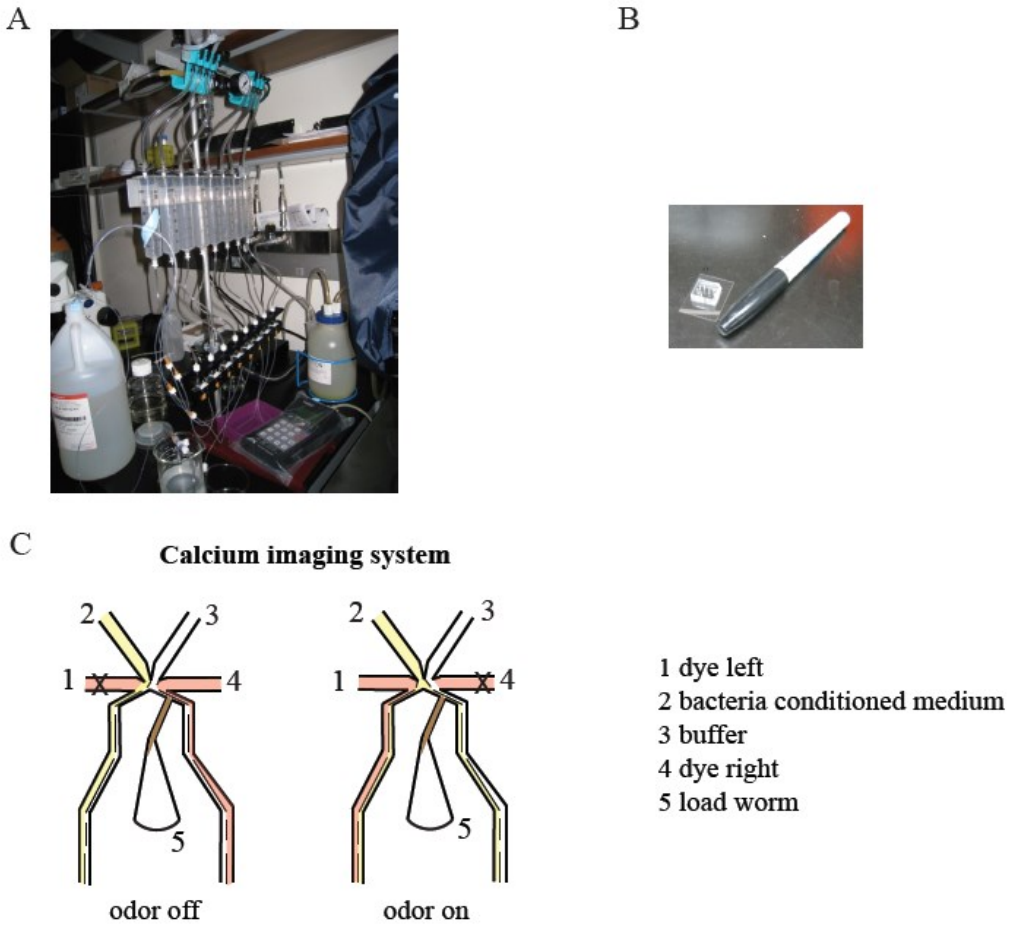


Figure 2.2 The calcium imaging system for *C. elegans* olfactory neuronal physiology study.

A, The olfactory stimuli delivery system. ***B,*** The size of the behavior chip. ***C,*** The scheme of the behavior chip.

I stimulated the transgenic animals with alternating fluid streams of buffer and the medium that was conditioned with the culture of *E. coli* OP50, the common laboratory bacterial food for *C. elegans*. I found that the ratio of YFP-to-CFP intensity increased when the stimulus switched from buffer to the conditioned medium, indicating increased intracellular calcium levels in response to the conditioned medium (Figure 2.3 A, B). Next, I examined whether the aversive training alters ADF activity by comparing the calcium responses of ADF neurons in the naive and trained animals. I found that trained animals exhibited calcium transients for an extended amount of time, ranging from 10 to 30 s (Figure 2.3 A, B), in response to the conditioned medium. To quantify this change, I calculated the time integral of the YFP/CFP ratio change during the last 20 s exposure to the conditioned medium and found that PA14-trained animals displayed a significant increase compared with naive animals (Figure 2.3 C). In contrast, training with PAK, a nonpathogenic strain of *P. aeruginosa*, did not induce similar changes ($p > 0.05$ for time integrals of the calcium transients over the last 20 s stimulus presentation, Student's ***t*** test) (Figure 2.3 D), suggesting that the training-dependent enhancement in ADF activity is contingent on the aversive experience with the pathogenic bacterium PA14. Together, these results indicate that the sensory-evoked calcium response in ADF is enhanced in the PA14-trained animals, suggesting that aversive training activates ADF neurons.

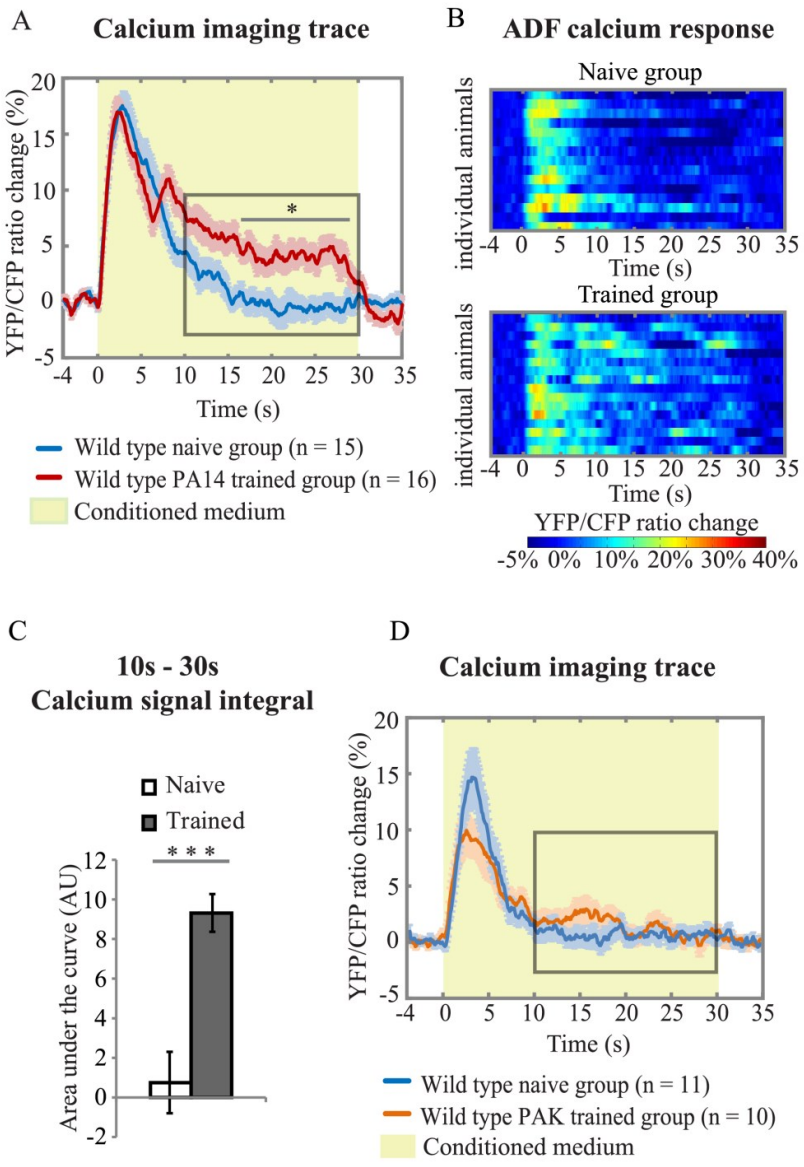


Figure 2.3 Calcium imaging of ADF neurons in naïve and trained conditions.

A, FRET signals of YC3.60 in ADF neurons in response to medium conditioned with *E. coli* OP50 in naive and PA14-trained wild-type animals. ***B,*** Heat maps for the FRET signals shown in ***A***. ***C,*** Time integral of the FRET signals in ***A*** during the last 20 s stimulus presentation. In ***A*** and ***C***, ***$p < 0.001$; *$p < 0.05$, Student's *t* test. Error bars represent SEM. ***D,*** FRET signals of YC3.60 in ADF neurons in response to OP50-conditioned medium in naive and PAK-trained wild-type animals.

To identify the source of ADF calcium transients, I examined the effects of several mutations that disrupt either intracellular calcium release from endoplasmic reticulumor calcium influx from extracellular space. First, I imaged ADF neurons in *itr-1(sa73)*and *unc-68(e540)* mutant animals, which carried a reduction-of-function mutation in the inositol-1,4,5-trisphosphate receptor ITR-1 (Dal Santo, Logan et al. 1999) and a splicing-acceptor site mutation in the ryanodine receptor UNC-68 (Sakube, Ando et al. 1997), respectively. I quantified the sensory-evoked ADF calcium transients by calculating the time integral of the YFP/CFP ratio change during the 30 s exposure to the conditioned medium, but I did not observe any obvious defect in ADF calcium responses to OP50-conditioned medium in these two mutants compared with wild-type animals ($p > 0.05$ for time integrals over 30 s stimulus exposure, Student's ***t*** test) (Figure 2.4 A, B). Because previous studies have indicated important roles of ITR-1 and UNC-68 in regulating intracellular calcium release (Maryon, Coronado et al. 1996; Sakube, Ando et al. 1997; Clandinin, DeModena et al. 1998; Baylis, Furuichi et al. 1999; Dal Santo, Logan et al. 1999; Busch, Laurent et al. 2012), these results suggest that intracellular calcium stores are not likely to be the main source for the sensory-evoked calcium transients in ADF.

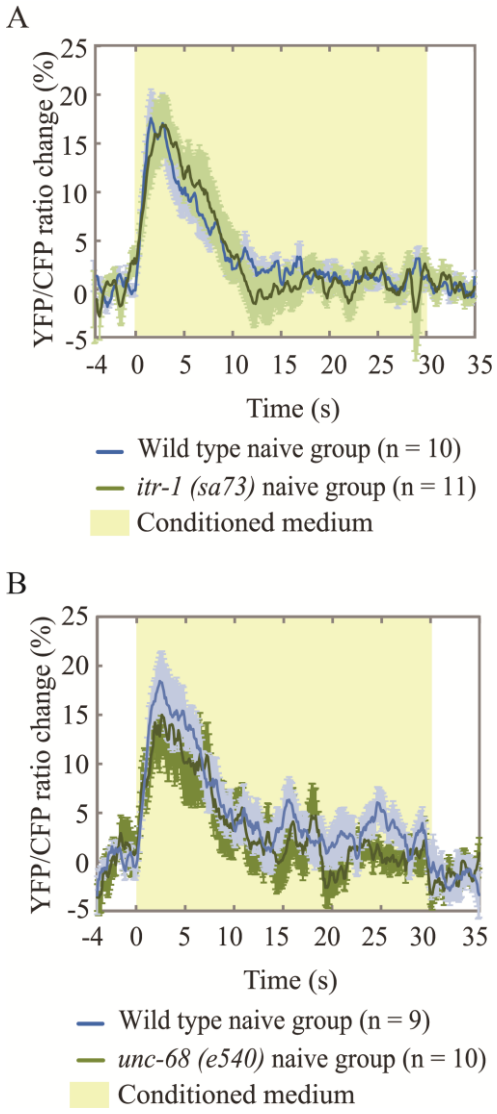


Figure 2.4 Calcium transients in ADF are not from the intracellular calcium stores.

A, FRET signals of YC3.60 in ADF neurons in response to OP50-conditioned medium in wild-type and *itr-1(sa73)* mutants. ***B,*** FRET signals of YC3.60 in ADF neurons in response to OP50-conditioned medium in wild-type and *unc-68(e540)* mutants. In ***A-B***, solid lines indicate mean values and shading indicates SEM.

Next, I tested ADF calcium response in *unc-2(e55)* animals, which carry a nonsense mutation in the *C. elegans* non-L-type voltage-gated calcium channel UNC-2 (Schafer and Kenyon 1995; Estevez, Estevez et al. 2004). I found that compared with wild-type animals, *unc-2(e55)* mutants displayed weaker ADF calcium transients in response to OP50-conditioned medium (Figure 2.5), and the integral of the calcium response during 30 s exposure of the stimulus is smaller in *unc-2(e55)* than in wild-type animals ($p < 0.05$, Student's *t* test). The partial defect of *unc-2(e55)* could be attributable to potential redundant roles played by other calcium channels. For example, the L-type voltage-gated calcium channel EGL-19 (Lee, Lobel et al. 1997) has been shown to regulate sensory-evoked calcium transients in several types of sensory neurons (Suzuki, Kerr et al. 2003; Hilliard, Apicella et al. 2005; Frokjaer-Jensen, Kindt et al. 2006). However, I could not assess the effect of *egl-19(ad1006)* mutants because of weak expression of Cameleon YC3.60 in the ADF neurons of the *egl-19* mutants. Both intracellular and extracellular sources of calcium contribute to the calcium responses of *C. elegans* neurons (Suzuki, Kerr et al. 2003; Hilliard, Apicella et al. 2005; Frokjaer-Jensen, Kindt et al. 2006; Busch, Laurent et al. 2012; Hendricks, Ha et al. 2012). These results together suggest that influx of extracellular calcium regulates the sensory-evoked calcium transients in ADF.

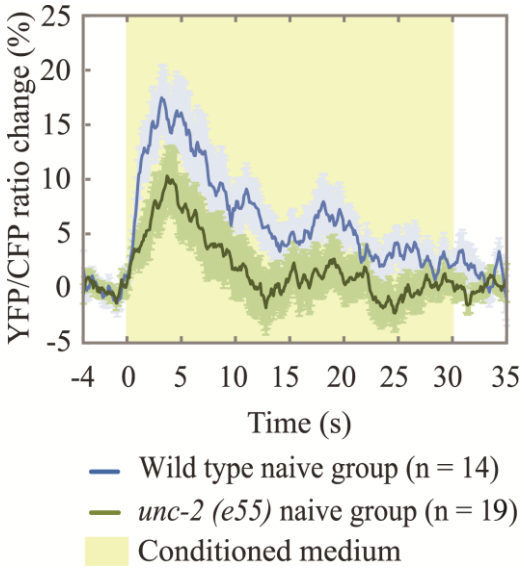


Figure 2.5 Calcium influx into the ADF neurons from extracellular space.

FRET signals of YC3.60 in ADF neurons in response to OP50-conditioned medium in wild-type and *unc-2(e55)* mutants. Solid lines indicate mean values and shading indicates SEM.

Changing ADF neuronal activity alters *tph-1* expression

Because aversive training increases both ADF calcium signal and *tph-1* transcription, I examined whether changing ADF neuronal activity can alter tph-1 transcription. First, I activated ADF neurons by selectively expressing a bacterially derived voltage-gated sodium channel, NaChBac (Ren, Navarro et al. 2001), in ADF. Ectopic expression of NaChBac depolarizes and increases the excitability of several types of neurons in flies and mice (Nitabach, Wu et al. 2006; Kelsch, Lin et al. 2009). I found that in comparison with non-transgenic siblings, the transgenic animals that expressed NaChBac only in ADF neurons displayed significantly enhanced *tph-1* expression in these neurons even in the absence of training (Figure 2.6 A), thus supporting the hypothesis that increased ADF neuronal activity during aversive training induces *tph-1* expression.

Next, I examined whether the training-dependent increase in *tph-1* transcription depends on ADF neuronal activity. I inhibited ADF neurons by selectively expressing an activated form of potassium channel *twk-18(cn110)* in ADF neurons. The expression of *twk-18(cn110)* in *Xenopus* oocytes generates a potassium channel that conducts significantly larger outward currents than the wild-type channels (Kunkel, Johnstone et al. 2000). I found that training-induced *tph-1* transcription was suppressed in the transgenic animals, whereas non-transgenic siblings displayed a normal level of induction (Figure 2.6 B). Together, these results support our hypothesis that activation of ADF neurons during the aversive experience leads to increased *tph-1* transcription.

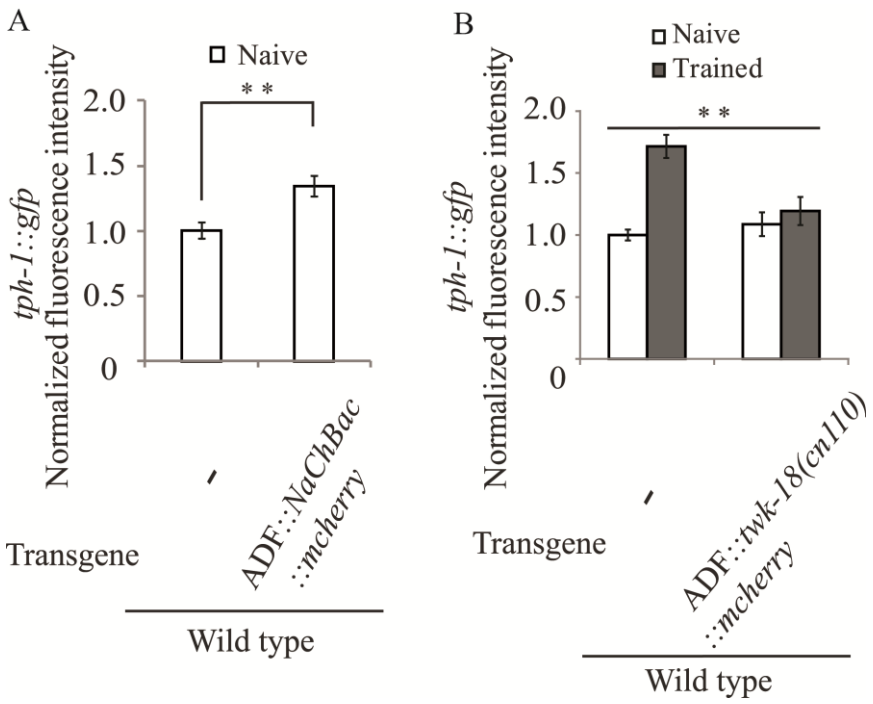


Figure 2.6 ADF *tph-1* transcription is dependent on ADF neuronal activity.

***A*,** Expression of a bacterially-derived voltage gated sodium channel NaChBac in ADF neurons enhances *tph-1* transcription. (** $p < 0.01$, Student's *t* test, $n \geq 50$ animals, mean ± SEM). ***B*,** Expression of a potassium channel encoded by *twk-18(cn110)* in ADF abolishes training-dependent increase in *tph-1* transcription. A significant genotype × treatment interaction (**$p < 0.01$) was detected by two-way ANOVA after log transformation ($p < 0.01$ for both genotype and treatment; $n \geq 30$ animals each sample). The data sets were tested as normal distribution by the Shapiro-Wilk test. Fluorescent signals were normalized by the signal of naive wild-type worms measured in parallel; error bars represent SEM.

CaMKII/UNC-43 functions in the ADF neurons to regulate *tph-1* transcription and aversive learning

Next, I sought the molecular mechanisms whereby *tph-1* transcription in ADF neurons is up-regulated by training experience. Because UNC-43, the only *C. elegans* homolog of calcium/calmodulin-dependent kinase type II (CaMKII) (Reiner, Newton et al. 1999; Rongo and Kaplan 1999) regulates ADF *tph-1* expression under normal conditions (Zhang, Sokolchik et al. 2004), I examined whether UNC-43 also regulates training-dependent *tph-1* expression in ADF. UNC-43 is a kinase with three domains, catalytic domain, regulatory domain and self-association domain. The binding between regulatory domain and catalytic domain regulates the activation state of the kinase (Figure 2.7).

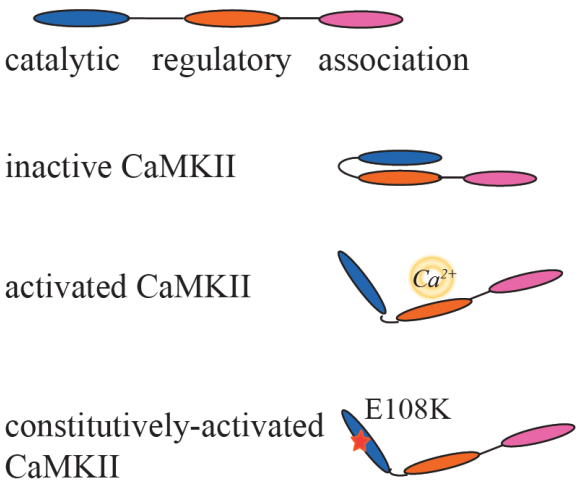


Figure 2.7 The structure and activation states of CaMKII.

First, I found that the training-dependent up-regulation of *tph-1* transcription in ADF neurons was defective in the *unc-43(n498n1186)* null mutants, measured by the expression of the transcriptional reporter *tph-1::gfp* in both naive and trained animals (Figure 2.8 A). In addition, the *unc-43(n498n1186)* mutants failed to learn to avoid the smell of PA14 after training (Figure

2.8). UNC-43 is ubiquitously expressed in the nervous system (Reiner, Newton et al. 1999; Rongo and Kaplan 1999). I found that selective expression of a wild-type *unc-43* cDNA in the ADF neurons of *unc-43(n498n1186)* mutants fully restored the training-dependent upregulation of *tph-1* expression in ADF (Figure 2.8 B) and partially rescued the learning defects in the mutants (Figure 2.9). One potential reason for the partial rescue of learning is that UNC-43 also regulates the function of motor neurons and muscles (Reiner, Newton et al. 1999; Rongo and Kaplan 1999), and the transgenic animals with UNC-43 activity restored only in ADF were still defective in locomotion. Thus, UNC-43 functions cell-autonomously in ADF neurons to regulate the training-dependent enhancement of *tph-1* transcription and aversive olfactory learning.

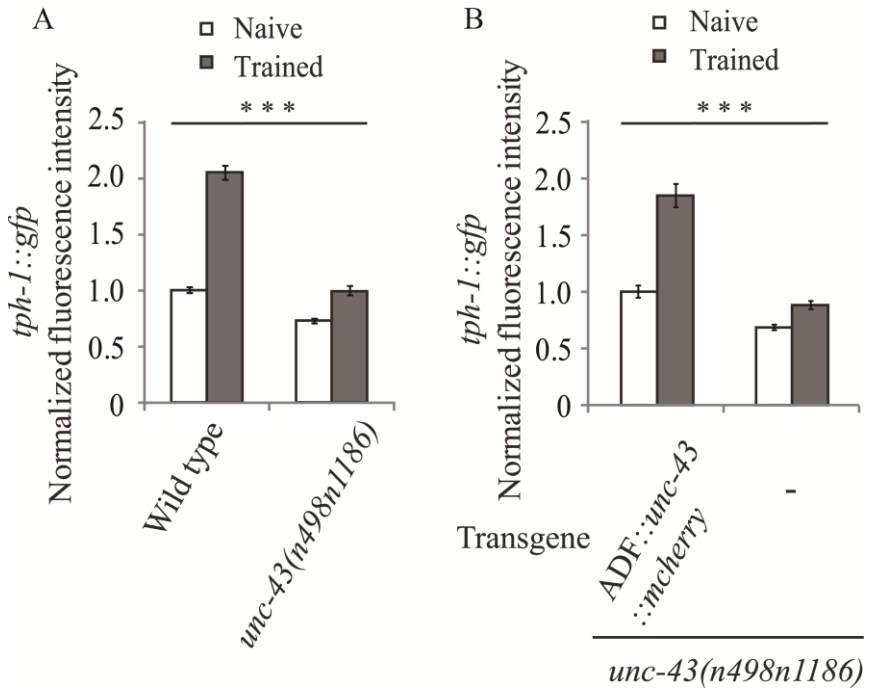


Figure 2.8 UNC-43 functions cell-autonomously in ADF neurons to regulate *tph-1* transcription.

A*,** The null mutant *unc-43(n498n1186)* is defective in training-dependent upregulation of *tph-1::gfp* expression in ADF neurons. ***B*,** Expression of *unc-43* in the ADF neurons of *unc-43(n498n1186)* mutants fully rescues the defect in *tph-1::gfp* regulation. The fluorescent signals were normalized by the signal of I wild-type worms **(*A*)** or I rescued animals **(*B*)** measured in parallel. Significant genotype × treatment interactions ($p < 0.001$) were detected by two-way ANOVA after log transformation ($p < 0.001$ for both genotype and treatment; $n \geq 59$ animals each sample). The data sets were tested as normal distribution by the Shapiro-Wilk test. Error bars represent SEM.

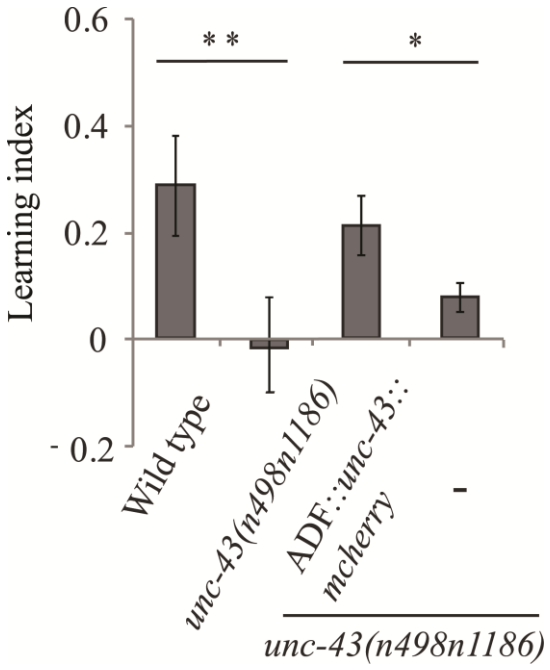


Figure 2.9 UNC-43 functions cell-autonomously in ADF neurons to regulate aversive olfactory learning.

The null mutant *unc-43(n498n1186)* is defective in learning to avoid the smell of PA14 and expression of *unc-43* in the ADF neurons of *unc-43(n498n1186)* mutants partially rescues the learning defect. $^{**}p < 0.01$, $^{*}p < 0.05$, Student's *t*-test, $n \geq 6$ assays. Error bars represent SEM.

The mammalian CaMKII isoform α_B is localized in the nucleus (Brocke, Srinivasan et al. 1995). However, I did not observe any obvious signal in the nuclei of transgenic animals that expressed a translational reporter *unc-43::mcherry* only in ADF neurons under either naive or trained condition (Figure 2.10 A, B). And it is important to notice that this *unc-43::mcherry* transgene is used to functionally rescue the mutant defect of *unc-43(n498n1186)* in *tph-1* and behavior regulation. The mammalian α_B-CaMKII possesses a nuclear localization signal (Brocke, Srinivasan et al. 1995) that is absent in UNC-43. This notion is consistent with the imaging results. Therefore, UNC-43 likely functions in the cytoplasmic and relay the signal into the nucleus through downstream signaling events to regulate *tph-1* transcription in ADF neurons.

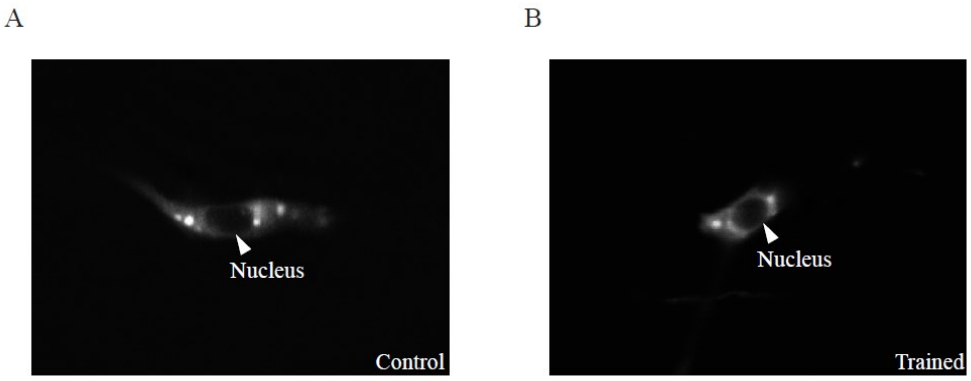


Figure 2.10 UNC-43 is localized in the cytoplasm in both the (control) condition (*A*) and the training condition (*B*).

Next, I investigated how UNC-43 functions cell-autonomously to regulate training-dependent *tph-1* transcription in ADF neurons. The *unc-43(n498)* mutant exhibits opposing phenotypes to the *unc-43* loss-of-function mutant in both locomotion and egg-laying (Reiner, Newton et al. 1999; Rongo and Kaplan 1999; Robatzek and Thomas 2000), and the amino acid substitution E108K in *unc-43(n498)* results in constitutive activation of UNC-43 that is calcium-independent (Umemura, Rapp et al. 2005) (Figure 2.7). First, I found that *tph-1* transcription in the ADF neurons of a gain-of-function mutant *unc-43(n498)* (Reiner, Newton et al. 1999; Rongo and Kaplan 1999) was significantly elevated under the I condition (Figure 2.10 A), consistent with previous studies (Zhang, Sokolchik et al. 2004). In addition, this elevated *tph-1* level was not further increased by training (Figure 2.11 A). These results suggest the possibility that activation of UNC-43 in ADF neurons during aversive training leads to increased *tph-1* expression. To further test this possibility, I selectively expressed the constitutively active form UNC-43(E108K) in the ADF neurons of the *unc-43(n498n1186)* null mutants and found that this ectopic expression enhanced *tph-1* transcription (Figure 2.11 B), compared with transgenic animals that expressed the wild-type UNC-43 in the ADF neurons of the same mutants. This enhancement strongly mimicked the training effect. The transgenic animals that expressed ADF-specific UNC-43(E108K) appeared to exhibit higher expression of *tph-1* in ADF neurons than the *unc-43(n498)* gain-of-function mutants. This difference may result from a higher expression level of UNC-43(E108K) in the ADF neurons of the transgenic animals and/or some unidentified defects in the *unc-43(n498)* mutant animals. Together, these findings support that activated UNC-43 in ADF neurons increases *tph-1* transcription during aversive training.

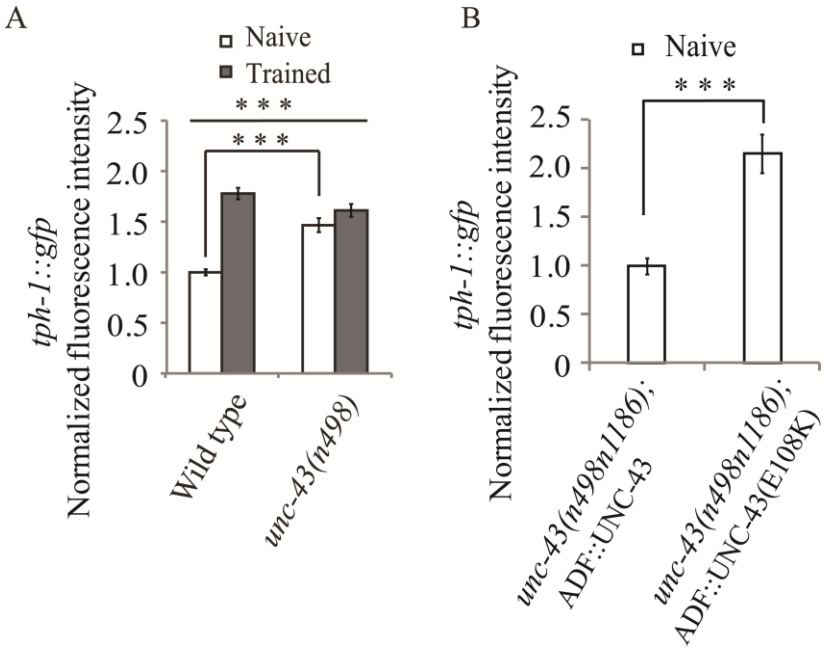


Figure 2.11 The *unc-43(n498)* mutation mimics the effect of training.

A*,** The gain-of-function mutant *unc-43(n498)* exhibits increased *tph-1::gfp* expression in ADF neurons in I animals ($p < 0.001$, Student's *t* test) and is defective in training-dependent increase in ADF *tph-1* expression [a significant genotype × treatment interaction (***$p < 0.001$) was detected by two-way ANOVA after log transformation; $p < 0.01$ for genotype, $p < 0.001$ for treatment; $n \geq 57$ each sample; error bars represent SEM; the data sets were tested as normal distribution by the Shapiro-Wilk test].

***B*,** *tph-1::gfp* expression in ADF neurons is enhanced in *unc-43(n498n1186)* mutants expressing UNC-43(E108K) in ADF neurons, compared with *unc-43(n498n1186)* mutants expressing wild-type UNC-43 in ADF. $P < 0.001$, Student's *t* test $n \geq 37$ animals; mean ± SEM.

CaMKII/UNC-43 regulates training-dependent enhancement of the ADF calcium responses

Having characterized the roles of UNC-43 and ADF neuronal activity in regulating *tph-1* transcription in ADF, I examined whether UNC-43 regulates ADF activity. I performed calcium imaging on the ADF neurons of *unc-43(n498n1186)* null mutants (Figure 2.12 A). I found that loss of UNC-43 activity abolished training-induced increase in ADF calcium transients and that this defect was fully rescued by selective expression of wild-type UNC-43 in the ADF neurons (Figure 2.12 A, B). Conversely, ADF-specific expression of the activated UNC-43(E108K) isoform in the *unc-43(n498n1186)* null mutants is sufficient to increase ADF calcium transients, mimicking the training effect (Figure 2.13 A, B). Moreover, training did not further enhance ADF calcium response in these transgenic animals. Together, these results indicate that aversive training experience activates ADF serotonergic neurons via activated function of UNC-43 in ADF, which leads to up-regulation of *tph-1* transcription.

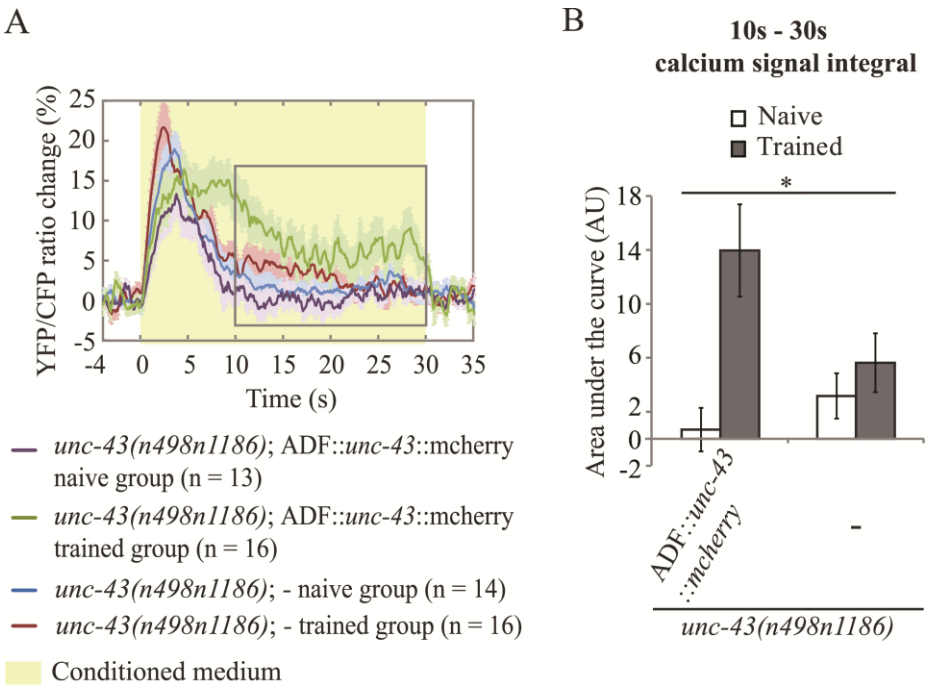


Figure 2.12 UNC-43 functions cell-autonomously in ADF neurons to regulate training-dependent increase in ADF neuronal activity.

***A*,** FRET signals of YC3.60 in ADF neurons in response to OP50-conditioned medium in *unc-43(n498n1186)* mutants and in the *unc-43(n498n1186)* mutant animals that selectively express wild-type UNC-43 in ADF under both I and PA14 training conditions. ***B*,** Time integral of FRET signals in *A* during the last 20 s stimulus presentation. A significant genotype × treatment interaction (*$p < 0.05$) was detected by two-way ANOVA ($p > 0.05$ for genotype, $p < 0.01$ for treatment; n ≥ 13 each sample; the data sets were tested as normal distribution by the Shapiro-Wilk test; error bars represent SEM).

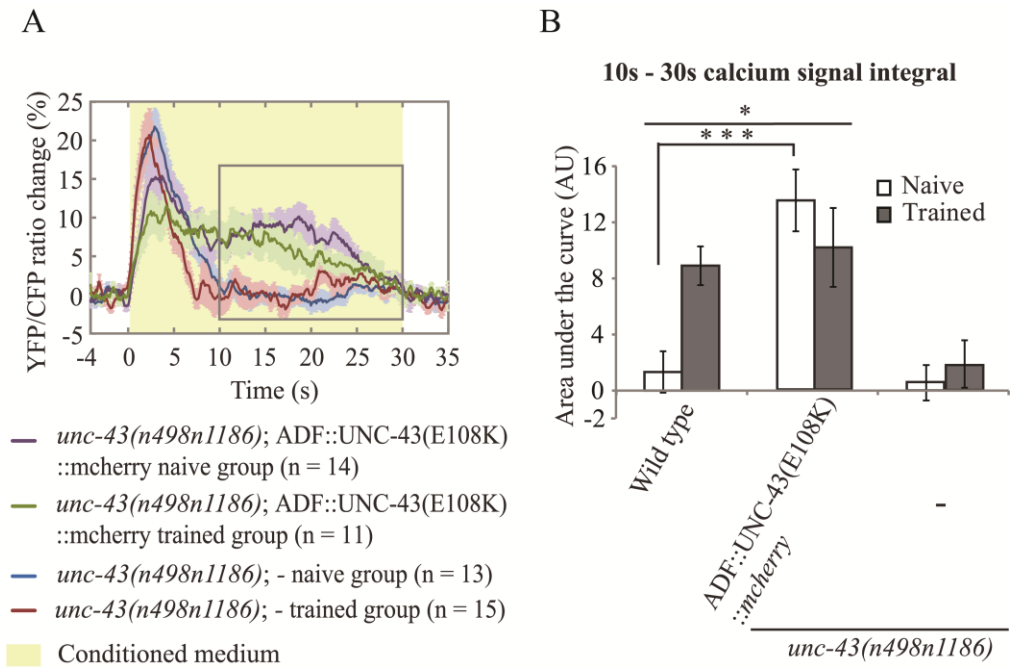


Figure 2.13 UNC-43 gain-of-function protein up-regulates ADF neuronal activity

A*,** FRET signals of YC3.60 in ADF neurons in response to OP50-conditioned medium in *unc-43(n498n1186)* mutants and in *unc-43(n498n1186)* mutants that selectively express the activated UNC-43(E108K) in ADF under both I and PA14-training conditions. ***B*,** Time integral of the FRET signals in ***A and of the FRET signals in wild-type animals during the last 20 s stimulus presentation. A significant genotype × treatment interaction (*$p < 0.05$) between wild-type and *unc-43(n498n1186)* animals that selectively expressed UNC-43(E108K) in ADF was detected by two-way ANOVA ($p < 0.01$ for genotype, $p > 0.05$ for treatment; n ≥ 11 each sample; the data sets were tested as normal distribution by the Shapiro-Wilk test). Student's *t* test was used to compare wild-type animals (n = 17) and *unc-43(n498n1186)* mutants that selectively expressed UNC-43(E108K) in ADF under the I condition (***$p < 0.001$). Error bars represent SEM.

Gq/EGL-30 regulates increased *tph-1* transcription during aversive training

Next, I investigated the molecular pathways in which UNC-43 acts to regulate tph-1 induction during training. Previously, it was shown that UNC-43 regulates locomotion and egg-laying through a pathway of GOA-1 and EGL-30, the *C. elegans* Goα and Gqα, respectively (Miller, Emerson et al. 1999; Robatzek and Thomas 2000; Lee, Jee et al. 2004). To test whether UNC-43 signals in the same pathway to regulate *tph-1* in ADF neurons during aversive learning, I analyzed the effect of mutations in the Goα/Gqα pathway on this process. Because mutants in this pathway are often deficient in locomotion, to ensure sufficient training I transferred trained animals from a standard training plate to a plate that was fully covered by a lawn of PA14 during the L4 larval stage and measured ADF tph-1 levels 12 hours later (Figure 1A).

First, I found that both *egl-30(ad805)* and *egl-30(n686)* (Brundage, Avery et al. 1996), two different mutations that disrupt EGL-30 function, abolished the training-dependent upregulation of *tph-1* transcription in ADF (Figure 2.14 A, B). Surprisingly, the gain-of-function mutant *egl-30(tg26)* (Doi and Iwasaki 2002; Bastiani, Gharib et al. 2003) is similarly defective (Figure 2.14 C), suggesting that proper activity level of Gqα/EGL-30 is important in regulating *tph-1* transcription. In contrast, a canonical mutant, *goa-1(sa734)*, exhibited normal training-dependent induction of *tph-1* despite an increased level of *tph-1* expression under the I condition (Figure 2.14 D). These results suggest that the functions of GOA-1 and EGL-30 in regulating training-dependent expression of *tph-1* are different from their antagonistic roles in locomotion and egg-laying and thus suggest a new mechanism of EGL-30 in regulating *tph-1* transcription.

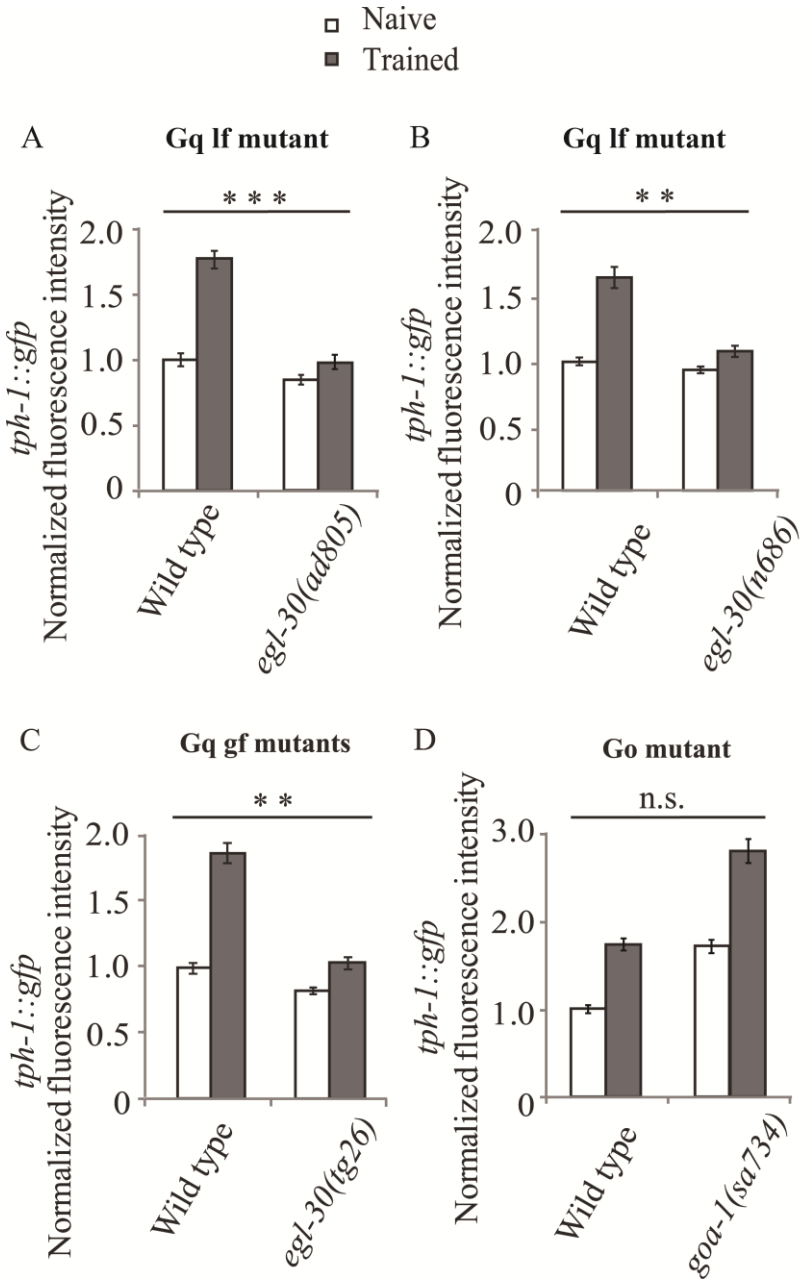


Figure 2.14 *egl-30 regulates tph-1* transcriptional induction in ADF neurons during training.

Figure 2.14 (continued)

A–C*,** The *egl-30(ad805)* **(*A*),** *egl-30(n686)* **(*B*),** and *egl-30(tg26)* **(*C*)** mutants are defective in *tph-1* transcriptional upregulation induced by aversive training; however, the *goa-1(sa734)* mutant animals **(*D*)** are normal in this training-dependent change. In ***A*, *B and ***D*** genotype × treatment interactions (***$p < 0.001$; **$p < 0.01$; n.s., $p \geq 0.05$) were tested by two-way ANOVA after log transformation ($p < 0.001$ for both genotype and treatment; $n \geq 40$ for each sample; the data sets were tested as normal distribution by the Shapiro-Wilk test). In ***C*,** a significant genotype × treatment interaction (**$p < 0.01$) was detected by the Scheirer-Ray-Hare nonparametric test ($p < 0.001$ for genotype and treatment; $n \geq 54$ animals for each sample), because the data sets were tested as deviated from normal distribution by the Shapiro-Wilk test. For all, the worms were trained with a modified protocol as in **Figure 1*A*.** The fluorescent signals were normalized by the signal of I wild-type worms measured in parallel. Error bars represent SEM.

Previous studies have implicated the Rho guanine nucleotide exchange factor (GEF) domain of UNC-73 (Williams, Lutz et al. 2007) and the phospholipase Cβ (PLCβ) EGL-8 as downstream effectors of EGL-30 in regulating locomotion and egg-laying (Lackner, Nurrish et al. 1999; Miller, Emerson et al. 1999). I found that a nonsense mutation in *egl-8(md1971)*, which produces strong defects in locomotion and egg-laying (Miller, Emerson et al. 1999), displayed normal training-dependent induction of *tph-1* expression in ADF neurons (Figure 2.15 A), suggesting that EGL-30 functions largely independently of EGL-8 to enhance *tph-1* transcription during aversive training. This result is consistent with a previous finding that EGL-8 acts as a minor downstream effector or in a parallel pathway of EGL-30 to regulate egg-laying behavior (Bastiani, Gharib et al. 2003). Similarly, the *unc-73(ce362)* mutant that contains a mutation in the Rho GEF domain was also not significantly defective in *tph-1* induction (Figure 2.15 B), implicating unidentified or novel effector(s) of Gqα/EGL-30 in regulating training-dependent *tph-1* transcription. Together, these results indicate that UNC-43 functions together with EGL-30 to modulate *tph-1* expression in ADF neurons during aversive training.

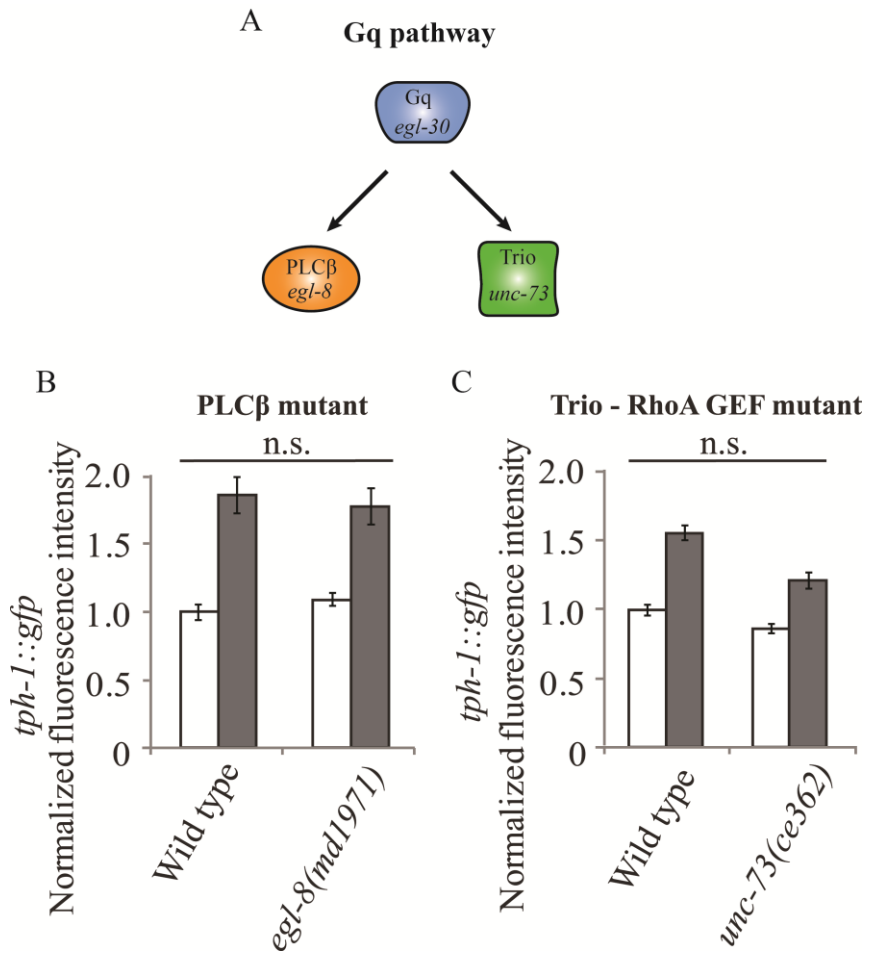


Figure 2.15 The downstream molecules of Gq pathway is differentially involved in *tph-1* regulation.

Figure 2.15 (continued)

***A*,** PLCβ and Trio are downstream molecules of Gq. The *egl-8(md1971)* mutants **(*B*)** and *unc-73(ce362)* mutants **(*C*)** are not significantly defective in training-dependent *tph-1* up-regulation in ADF neurons. For ***B*,** genotype × treatment interaction was tested by two-way ANOVA after log transformation (n.s., $p > 0.05$ for interaction, $p = 0.902$ for genotype, and $p < 0.001$ for treatment; $n \geq 40$ for each sample). For ***C*,** genotype × treatment interactions were tested by two-way ANOVA after log transformation (n.s., $p > 0.05$ for interaction, $p < 0.001$ for both genotype and treatment; $n \geq 40$ for each sample). For all, the data sets were tested as normal distribution by the Shapiro-Wilk test the worms were trained with a modified protocol as in **Figure 1A**. The fluorescent signals were normalized by the signal of I wild-type worms measured in parallel. Error bars represent SEM.

Gq/EGL-30 mediates sensory response of AWB and AWC olfactory neurons to regulate *tph-1* induction during aversive training

Next, I examined the mechanisms whereby EGL-30 regulates *tph-1* transcription in ADF neurons during aversive training. *Egl-30* is known to express widely in the nervous system (Lackner, Nurrish et al. 1999). First, I confirmed the expression of *egl-30* in ADF neurons (Figure 2.16 A) and the major olfactory sensory neurons AWB and AWC (Figure 2.16 B) by examining the co-expression of a transcriptional reporter KP326 (*Pegl-30::gfp*) (Lackner, Nurrish et al. 1999) with mCherry reporters expressed in these neurons.

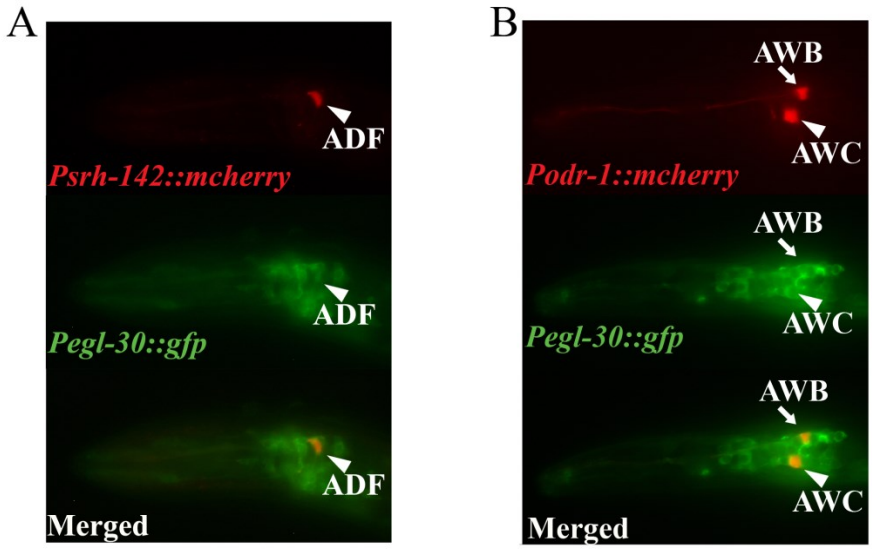


Figure 2.16 *egl-30* is expressed in ADF, AWB and AWC neurons.

A*, *egl-30 is expressed in the ADF neurons, as shown by colocalization of the fluorescent signals generated by the *Psrh-142::mcherry* and the *Pegl-30::gfp* transcriptional reporters. ***B*, *egl-30*** is expressed in the AWB and AWC neurons, as shown by colocalization of the fluorescent signals generated by the *Podr-1::mcherry* and the *Pegl-30::gfp* transcriptional reporters.

Next, I found that, different from the cell-autonomous role of UNC-43, selective expression of a wild-type *egl-30* cDNA in ADF neurons did not rescue the defect of *egl-30(ad805)* mutants in regulating training-dependent induction of *tph-1* expression in ADF (Figure 2.17 A). AWB synapse onto ADF with multiple chemical synapses (White, Southgate et al. 1986), but expression of *egl-30* in AWB alone did not rescue (Figure 2.17 B). This defect and the similar defect in the other *egl-30* mutant, *n686*, required the activity of *egl-30* in both AWB and AWC sensory neurons to rescue (Figure 2.18 A, B). Together, these results reveal a small neuronal network in which *unc-43* functions cell-autonomously in ADF neurons and *egl-30* functions non-cell-autonomously in the upstream sensory neurons to produce activity-dependent up-regulation of *tph-1* transcription in ADF.

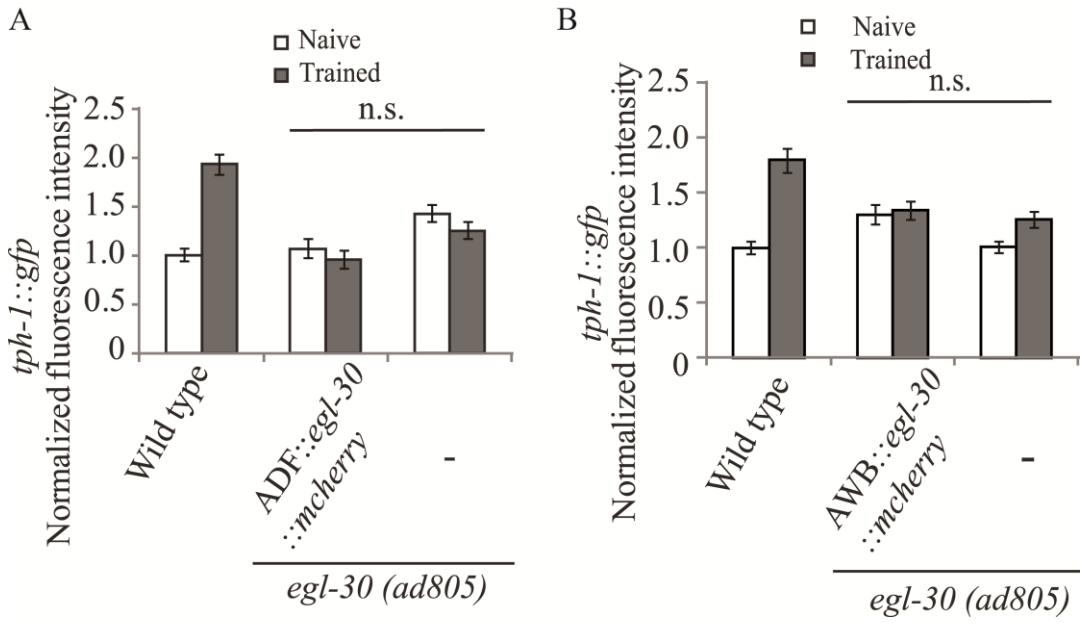


Figure 2.17 Expression of *egl-30* in ADF or AWB neurons cannot rescue the mutant defect.

Figure 2.17 (continued)

***A*,** Expression of *egl-30* in ADF neurons does not rescue the mutant phenotype of *egl-30(ad805*) in *tph-1* expression. Two-way ANOVA after log transformation, n.s. $p > 0.05$ for genotype × treatment interaction, $p < 0.001$ for genotype, $p = 0.07$ for treatment; $n \geq 28$ for each group; the data sets were tested as normal distribution by the Shapiro-Wilk test. Error bars represent SEM. .

***D*,** Expression of *egl-30* in AWB neurons alone does not rescue the mutant phenotype of *egl-30(ad805)* in *tph-1* expression. The genotype × treatment interaction (n.s., $p > 0.05$) was tested by the Scheirer-Ray-Hare nonparametric test ($p = 0.12$ for genotype, $p < 0.05$ for treatment; $n \geq 40$ for each sample), because the data sets were tested as deviated from normal distribution by the Shapiro-Wilk test. Error bars represent SEM.

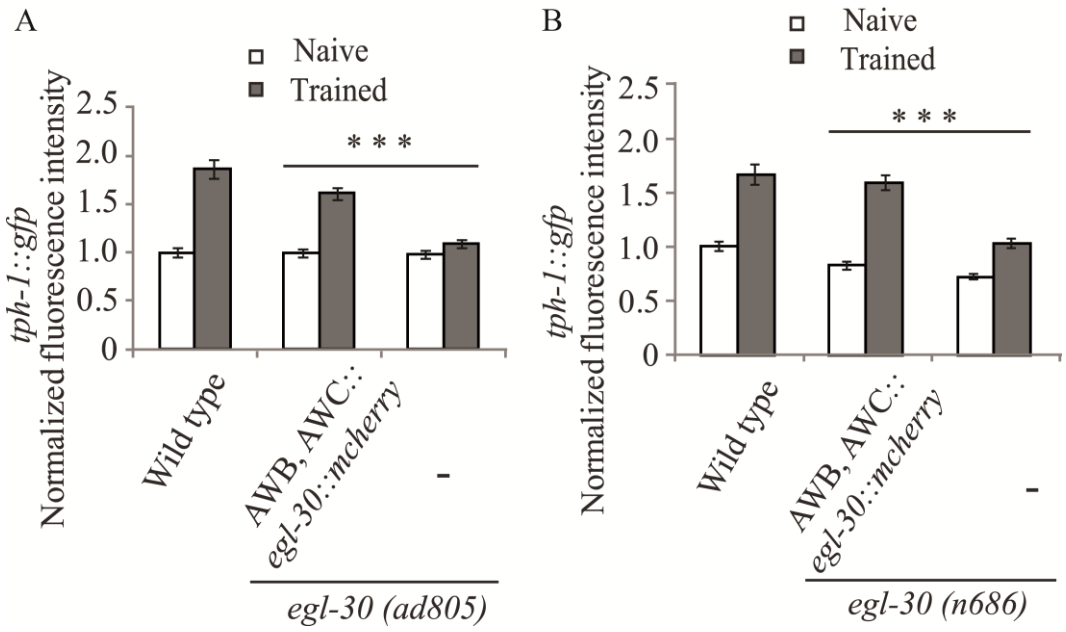


Figure 2.18 EGL-30 functions in the AWB and AWC sensory neurons to regulate training-dependent induction oftph-1transcription in ADF neurons.

Expression of *egl-30* in both AWB and AWC fully rescues the defect of*egl-30(ad805)* **(*A*)** and *egl-30(n686)* **(*B*)** in *tph-1* regulation. A significant genotype × treatment interaction (***$p < 0.001$) was detected by the Scheirer-Ray-Hare nonparametric test **(*A*)** or by two-way ANOVA after log transformation **(*B*),** $p < 0.001$ for genotype and treatment **(*A, B*)**; $n \geq 35$ for each group; the data sets were tested as deviated from normal distribution **(*A*)** or normal distribution **(*B*)** by the Shapiro-Wilk test. Error bars represent SEM.

Consistently, ADF-specific expression of the activated UNC-43(E108K) in *egl-30(ad805)* mutants significantly increases ADF *tph-1* expression (Figure 2.19), further supporting the possibility that UNC-43 acts downstream of EGL-30 in regulating ***tph-1*** expression in ADF.

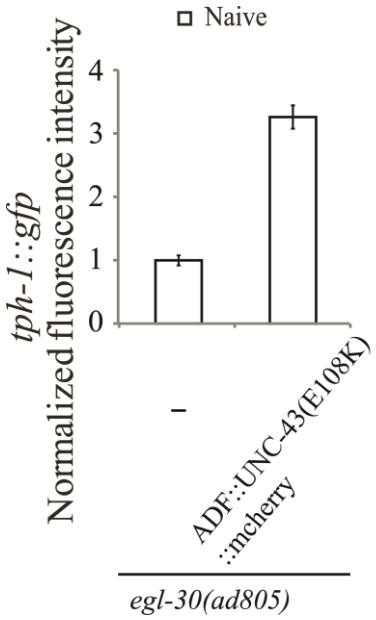


Figure 2.19 Expression of UNC-43(E108K) in ADF neurons in the *egl-30(ad805)* background also enhances *tph-1* transcription in the absence of PA14 training.

Student's *t* test, $p < 0.001$ between transgenic animals and nontransgenic siblings; *tph-1* signals were normalized by the signals in nontransgenic animals; $n \geq 25$ animals each group; mean ± SEM.

Next, I investigated the function of *egl-30* in AWB and AWC sensory neurons. First, I found that expression of *egl-30* in AWB and AWC neurons did not significantly alter locomotory speed (transgenic animals, 0.13 ± 0.04 pixels s^{-1}; nontransgenic siblings, 0.27 ± 0.13 pixels s^{-1}; Student's *t* test, $p > 0.05$; $n = 5$ animals each group; mean ± SEM), suggesting that *egl-30* function in these neurons is not essential for locomotion. Using slow-killing assays (Kawli, Wu et al. 2010) to measure resistance to PA14 infection, I found that the activity of *egl-30* in AWB and AWC also did not significantly change the susceptibility of the *egl-30(n686)* mutants (Figure 2.20), consistent with the previous finding on the role of intestinal EGL-30 in regulating pathogen resistance (Kawli, Wu et al. 2010).

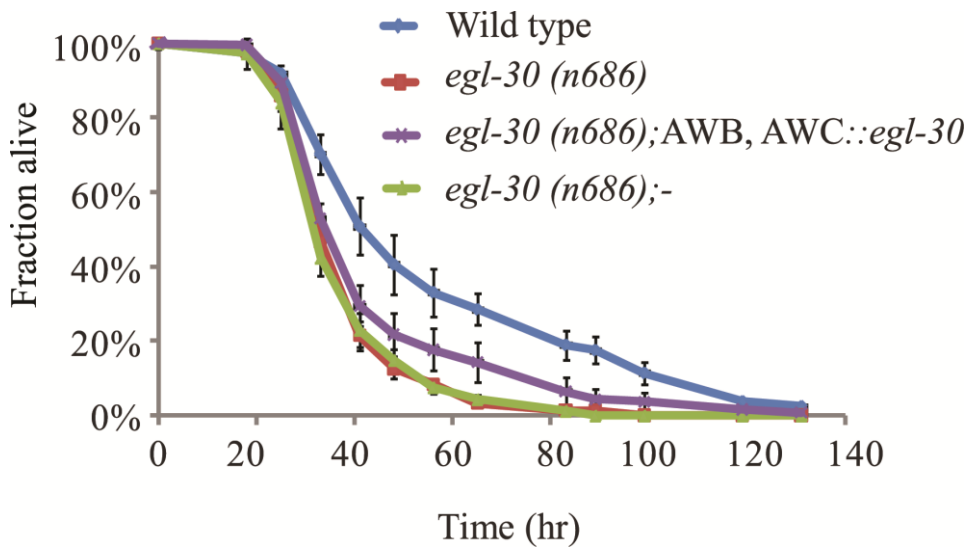


Figure 2.20 The resistance of *egl-30(n686)* mutants to PA14 is not significantly rescued by the expression of *egl-30* in AWB and AWC neurons.

(*n* = 3 assays, 3 plates per assay, no significant difference among the *egl-30(n686)* groups, the difference between wild-type and all other genotypes is significant, log-rank test with Bonferroni correction; error bars represent SEM).

Finally, I examined whether *egl-30* mediates the sensory response of AWB and AWC, the major sensory neurons that detect repellents and attractants, respectively (Bargmann, Hartwieg et al. 1993). The sensory ability of these two pairs of neurons is essential for worms to learn to avoid the smell of pathogenic bacteria (Ha, Hendricks et al. 2010), and both AWB and AWC cells are activated by the removal of medium conditioned by the worm food *E. coli* OP50 (Chalasani, Chronis et al. 2007; Ha, Hendricks et al. 2010).

Using intracellular calcium imaging and transgenic animals that selectively expressed the calcium-sensitive fluorescent protein GCaMP (Nakai, Ohkura et al. 2001) or GCaMP2.0 (Tallini, Ohkura et al. 2006), I found that in comparison with wild-type animals, *egl-30(ad805)* mutants displayed a significantly weaker calcium response in AWB neurons upon the removal of OP50-conditioned medium (Figure 2.21A). This defect was fully rescued by selective expression of *egl-30* cDNA in AWB neurons (Figure 2.21 B).

Similarly, *egl-30(ad805)* mutants showed a mild defect in the calcium response of AWC neurons (Figure 2.22). Together, these results indicate that EGL-30 functions in AWB and AWC neurons to regulate olfactory sensory response, suggesting an important role of sensory experience in the upregulation of *tph-1* transcription during aversive training.

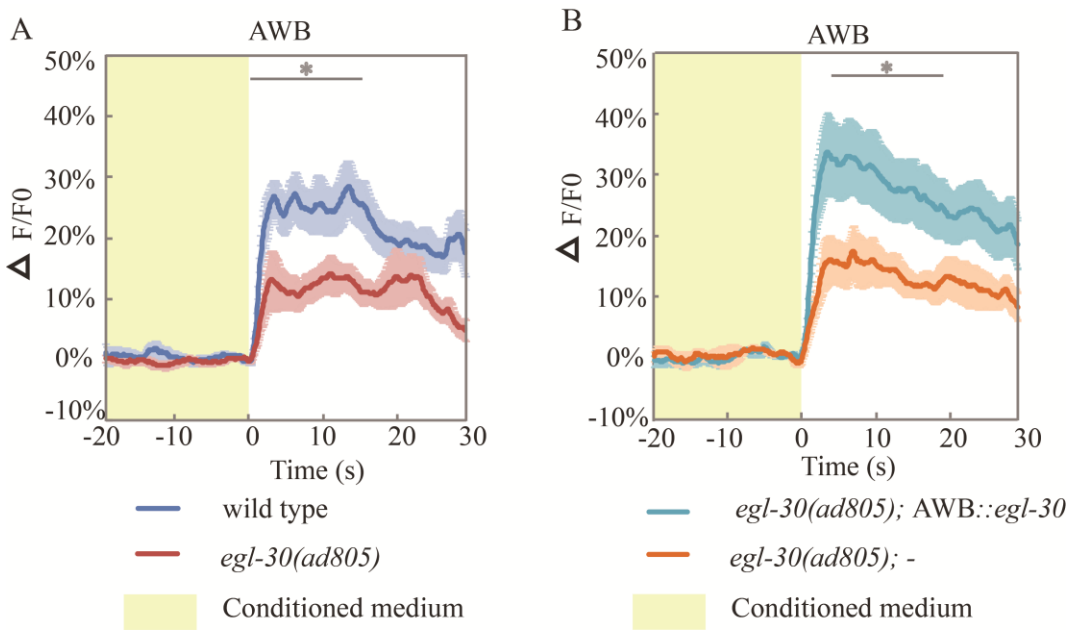


Figure 2.21 EGL-30 mediates the sensory function of the AWB olfactory neurons to regulate training-dependent increase in *tph-1* transcription in ADF neurons

GCaMP signals of AWB neurons in wild-type and *egl-30(ad805)* mutant animals **(*A*)** and in transgenic animals that express *egl-30* selectively in AWB and nontransgenic siblings **(*B*).** Solid lines indicate mean, and shading indicates SEM. **p* < 0.05, Student's *t* test.

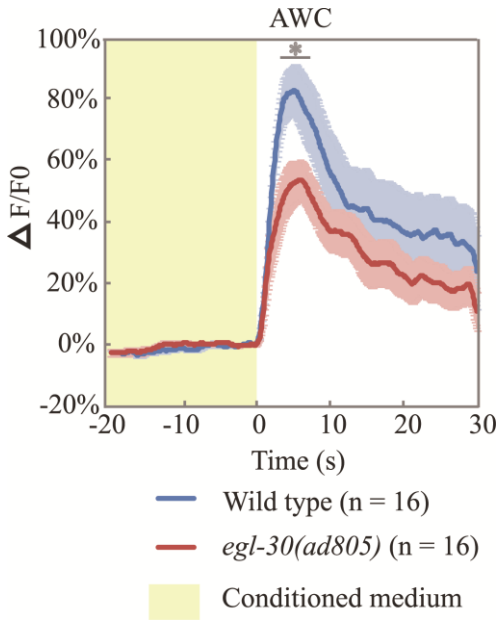


Figure 2.22 EGL-30 mediates the sensory function of the AWC olfactory neurons to regulate training-dependent increase in *tph-1* transcription in ADF neurons

GCaMP2 signals of AWC neurons in wild-type and *egl-30(ad805)* mutant animals. Solid lines indicate mean, and shading indicates SEM. **p* < 0.05, Student's *t* test.

AWB and AWC neurons may signal to ADF neurons through peptide signaling

Based on the EM data, AWB neurons have direct synaptic connections with ADF neurons, while AWC neurons do not have direct synaptic connections. It would be interesting to ask how Gq in AWB and AWC neurons transmit the sensory information to ADF neurons. To obtain preliminary data to answer this question, I tested *tph-1* transcription up-regulation induced by pathogenic bacterial training in *unc-13(e51)* and *unc-31(e928)* mutants. *unc-13* encodes for a protein that interacts with syntaxin to regulate synaptic vesicle fusion and thus is required for synaptic vesicle exocytosis (Madison, Nurrish et al. 2005). In *unc-13(e51)* mutant, *tph-1* transcription level is increased after training as the level of wild type, which suggests that information transmitted through canonical synaptic connection is not essential for the signal from AWB to ADF neurons (Figure 2.23 A). *unc-31* encodes for the *C. elegans* ortholog of human calcium-dependent activator protein for secretion (CAPS), which is responsible for the exocytosis of dense-core vesicle release (Speese, Petrie et al. 2007). *unc-31(e928)* mutant is defective in *tph-1* transcription upregulation induced by the training, suggesting that signal transmitted through dense-core vesicle such as neuropeptides may be responsible for the information transmitted from AWB, AWC neurons to ADF neurons (Figure 2.23 B).

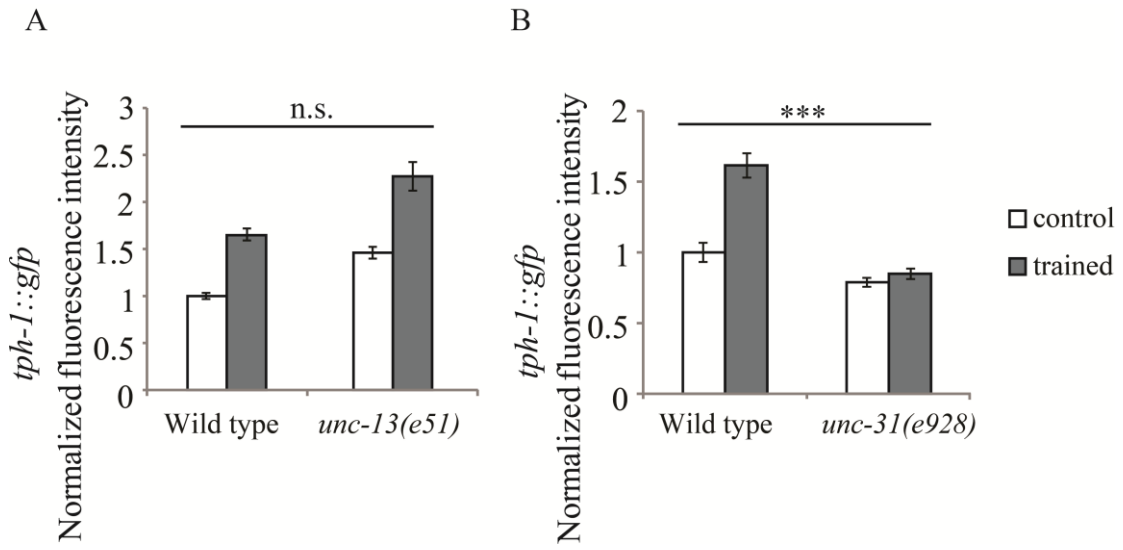


Figure 2.23 AWB, AWC neurons may signal to ADF through peptide signaling.

A*, *unc-13(e51) mutant is not defective in training-dependent *tph-1* transcription upregulation. ***B*, *unc-31(e928)*** mutant is defective in training-dependent tph-1 transcription upregulation. (n.s. not significant, *** $p < 0.001$, genotype treatment interaction tested by 2-way Anova).

Discussion

Regulated expression of tryptophan hydroxylase (TPH), the rate-limiting biosynthetic enzyme of serotonin, is essential for serotonergic signaling. In this chapter, I characterize a neuronal signaling network that regulates the experience-dependent transcription of *C. elegans tph-1* during aversive olfactory learning. I show that aversive training activates ADF neurons and that increased ADF neuronal activity results in enhanced *tph-1* expression. This experience-dependent increase in ADF activity and *tph-1* expression requires the function of UNC-43 in ADF. ADF-selective expression of an activated form of UNC-43 produces the training effect on ADF neuronal activity and *tph-1* transcription in the absence of training. I also show that Gqα/EGL-30 regulates the sensory function of AWB and AWC olfactory neurons, the sensory neurons upstream of ADF, to mediate the training-dependent enhancement of *tph-1* expression, implicating the importance of sensory experience. Thus, this chapter provides mechanistic insights into experience-dependent regulation of *tph-1* expression.

Intracellular calcium signals regulate serotonergic signaling

L-type voltage-gated calcium channel EGL-19 regulates sensory-evoked calcium transients in several *C. elegans* mechanosensory neurons (Suzuki, Kerr et al. 2003; Frokjaer-Jensen, Kindt et al. 2006), nociceptive neuron ASH (Hilliard, Apicella et al. 2005), and oxygen sensing neurons, such as URX (Busch, Laurent et al. 2012). Although I could not assess the role of *egl-19* in ADF calcium transients due to weak expression of the calcium indicator Cameleon YC3.60 in *egl-19(ad1006)* mutants, I have identified the role of UNC-2, a non-L-type voltage-gated calcium channel, in regulating sensory-evoked calcium response in ADF. Previous studies have shown that mutations in *unc-2* decrease *tph-1* expression in ADF (Estevez, Estevez et al. 2004).

Together, these results suggest that UNC-2-mediated intracellular calcium level plays an important role in transcriptional regulation of *tph-1* in ADF neurons.

Intracellular calcium levels are known to regulate serotonergic signaling. In mammals, an increase in the intracellular calcium concentration in brain tissues leads to an increase in serotonin synthesis (Elks, Youngblood et al. 1979; Hamon, Bourgoin et al. 1979). Here, I found that the sensory-evoked calcium response of ADF serotonergic neurons is enhanced after aversive training and increased ADF neuronal activity generates elevated transcription of *tph-1*. Thus, I propose that activity-dependent regulation of *tph-1* transcription is one of the mechanisms that underlie calcium-regulated serotonin production. In rodents, repeated immobilization stress, repeated forced swimming stress and chronic variable stress all can elevate TPH2 mRNA level (Chamas, Serova et al. 1999; Chamas, Underwood et al. 2004); (Shishkina, Kalinina et al. 2008); (McEuen, Beck et al. 2008). The increased serotonergic neuronal activity may underlie the experience-dependent regulation of TPH in these scenarios.

The mechanisms and consequences of enhanced intracellular calcium signal

Experience can modulate neuronal calcium signaling. For example, in the aversive olfactory learning paradigm of *Drosophila* where odors are paired with electric shocks, the odor-evoked calcium response of the serotonergic DPM neurons increases after training (Yu, Keene et al. 2005; Lee, Lin et al. 2011). Similarly, I observed that the calcium signal in the ADF serotonergic neurons in response to bacterial odor is enhanced after training with the pathogenic bacteria PA14. These changes in calcium dynamics can be achieved by modulating both extracellular and intracellular sources. For example, the activity of L-type calcium channels can be enhanced by the phosphorylation of PKA, resulting in the increased calcium current mediated by these channels (Gray and Johnston 1987; Gross, Uhler et al. 1990; Wang and Guan 2010). The calcium

channels on endoplasmic reticulum (ER) can also be modulated (Berridge 1998). For instance, IP3 receptor can be sensitized through several mechanisms, which lowers the threshold for activation of the receptor by IP3 and increases the calcium release from ER (Dudek 2008; Ahn, Bernier et al. 2010; Kew and Davies 2010; Tovey, Dedos et al. 2010). Similarly, ryanodine receptors (RYR) can also be sensitized by the putative second messenger cyclic ADP ribose in cultured neurons (Hua, Tokimasa et al. 1994). In this chapter, I demonstrated that non-L-type voltage-gated calcium channel UNC-2 is one of the source for ADF calcium transients. The enhanced ADF calcium signaling could be from enhanced function of UNC-2, it could also be from enhanced function of other intracellular or extracellular channels. To further answer this question, calcium imaging experiments will need to be done in trained animals, looking for defect in these mutant animals.

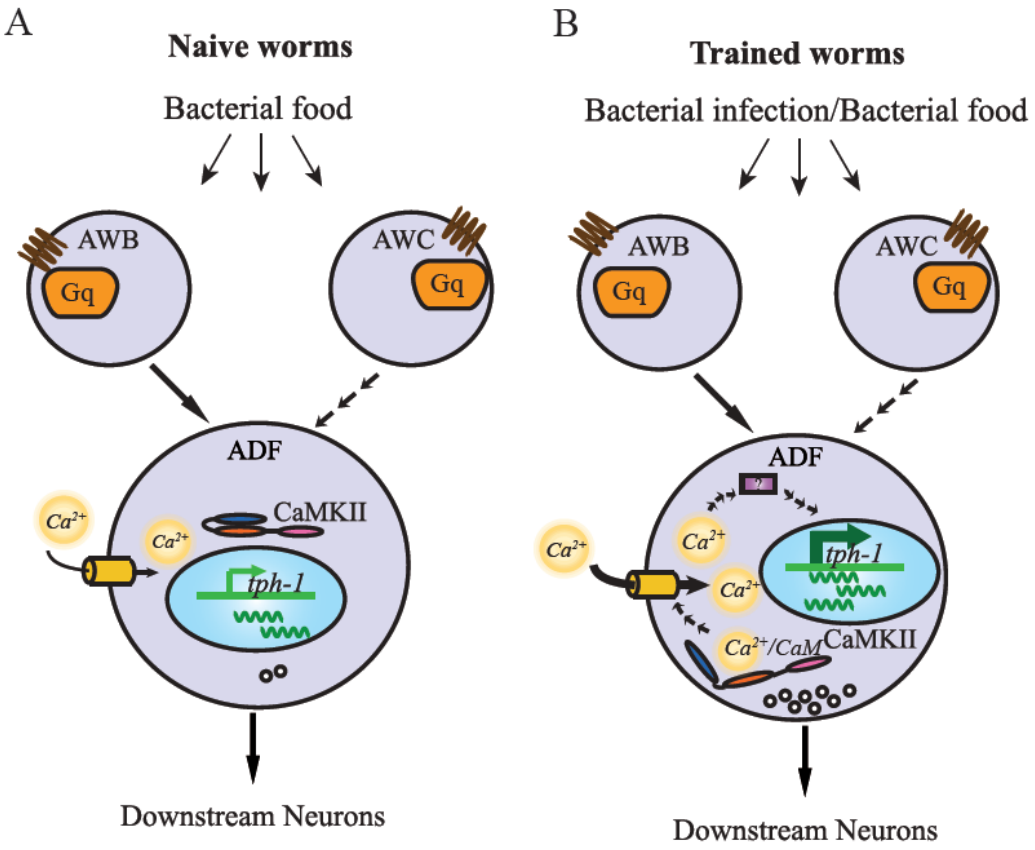
Elevated intracellular calcium level can alter many neuronal processes, including exocytosis. The fast local calcium elevation resulted from activation of voltage-sensitive channels near the active zone may regulate the release of neurotransmitters in synaptic vesicles, while the global and relatively slower calcium release from internal stores may trigger the release of neuropeptides from dense core vesicles (Verhage, McMahon et al. 1991; Peng 1996). The release of amines seems to be regulated by calcium levels that are intermediate (Verhage, McMahon et al. 1991). In addition, increased calcium signals can activate gene transcription, as is demonstrated in this chapter and other literatures (Flavell and Greenberg 2008).

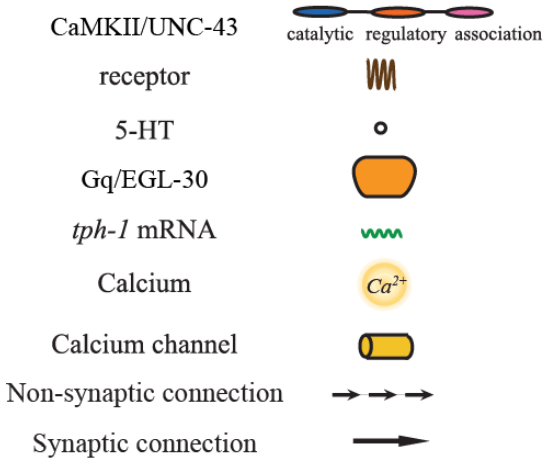
CaMKII enhances serotonergic neuronal calcium signaling to induce TPH transcription

Importantly, in this chapter, I have identified a cell-autonomous role of CaMKII/UNC-43 in regulating training-dependent activity increase in ADF neurons. I have also shown that increased ADF activity enhances *tph-1* transcription. Thus, I propose that aversive training experience up-

regulates *tph-1* transcription in ADF neurons via UNC-43-mediated neuronal activity enhancement (Figure 2.24). In the mammalian serotonergic system, TPH can be directly phosphorylated and activated by CaMKII (Ehret, Cash et al. 1989). Here, I show that CaMKII also enhances *tph-1* transcription, providing a new mechanism whereby CaMKII regulates serotonin signaling. Previous studies have shown that UNC-43 is required to maintain the basal level of *tph-1* transcription under normal conditions (Estevez, Estevez et al. 2004; Zhang, Sokolchik et al. 2004; Estevez, Cowie et al. 2006). Data presented in this chapter reveal several novel functions of UNC-43 in regulating *tph-1*. First, UNC-43 regulates *tph-1* transcription in a way that is dependent on training experience. Second, UNC-43 regulates the training-dependent change in ADF activity and *tph-1* transcription in a cell-autonomous manner, linking the signal transduction of UNC-43 and *tph-1* transcription within the same cells. Third, ADF-specific expression of a constitutively active form of UNC-43 mimics the training effect on ADF neuronal activity and *tph-1* transcription, suggesting that activation of UNC-43 in ADF is a functional consequence of training that leads to increased ADF neural activity and *tph-1* expression. Meanwhile, increased ADF neuronal activity, measured by intracellular calcium levels, leads to increased *tph-1* transcription, causally linking the UNC-43-mediated neuronal activity change with increased *tph-1* transcription within ADF ("UNC-43 → neuronal activity → *tph-1* transcription"). During hippocampal long-term potentiation, high-frequency stimulation activates postsynaptic CaMKII, which in turn alters neuronal activity by modulating the conductance and localization of ionotropic receptors (Lisman, Yasuda et al. 2012). Here I propose that training experience activates the worm CaMKII, UNC-43, in ADF neurons, which enhances ADF neuronal activity by increasing intracellular calcium levels. In the mammalian nervous system, elevated intracellular calcium levels activate kinase pathways, including those

of PKA, CaMK and RSK/MSK, which results in transcriptional changes via modulation of transcriptional regulators (Flavell and Greenberg 2008). This chapter suggests that the signal of UNC-43-mediated calcium increase in trained ADF neurons is conveyed into the nucleus to enhance *tph-1* transcription (Figure 2.24).





The working model proposes that:

1. Training experience activates the worm CaMKII, UNC-43, in the ADF neurons, which enhances ADF neuronal activity by increasing intracellular calcium levels.

2. The signal of UNC-43-mediated calcium increase in trained ADF neurons is conveyed into the nucleus to enhance *tph-1* transcription.

3. EGL-30, the worm Gqα mediates sensory responses of the upstream olfactory sensory neurons, to induce *tph-1* transcription during training.

Figure 2.24 Working model that describes the role of CaMKII/UNC-43 and Gq/EGL-30 signaling pathway in regulating *tph-1* transcription in ADF neurons in the naive (A) and trained (B) animals.

CaMKII and Gq function in a non-canonical pathway to regulate *tph-1* transcription

CaMKII is a calcium-activated enzyme that plays an essential role in calcium signaling and regulates activity-dependent changes in a variety of neuronal functions (Lisman, Schulman et al. 2002). Mutations in *unc-43,* which encodes the *C. elegans* CaMKII, generate pleiotropic defects, including abnormal locomotion, egg-laying and defecation (Reiner, Newton et al. 1999; Rongo and Kaplan 1999; Robatzek and Thomas 2000), and UNC-43 regulates these different behaviors through different effectors. Particularly, in locomotion and egg-laying, UNC-43 acts with a Goα (GOA-1) and a Gqα (EGL-30), which play antagonistic roles (Mendel 1995; Segalat, Elkes et al. 1995; Lackner, Nurrish et al. 1999; Miller, Emerson et al. 1999; Nurrish, Segalat et al. 1999; Robatzek and Thomas 2000; Lee, Jee et al. 2004). Here, I show that GOA-1 is not essential for the training-induced *tph-1* up-regulation in ADF neurons whereas both UNC-43 and EGL-30 are required for this regulation. Furthermore, prior to this study, it was not clear whether UNC-43 and EGL-30 act in the same cells for their signaling functions. Here, I show that UNC-43 plays a cell-autonomous role in ADF neurons, whereas EGL-30 plays a non-cell-autonomous role in the upstream olfactory sensory neurons, to induce *tph-1* transcription during training. Thus, this chapter reveals another mechanism for the diverse function of the UNC-43 and EGL-30 signaling pathway.

Two Gα proteins, Gqα and ODR-3, mediate sensory response in olfactory sensory neurons.

Data presented in this chapter also identify an unexpected role of Gqα/EGL-30 in mediating the sensory response of olfactory neurons. Olfactory sensation in vertebrates is conveyed by G proteins. Binding of odors with G-protein-coupled odorant receptors (GPCRs) activates Gsα-like G proteins to initiate downstream signaling (Jones and Reed 1989; Belluscio, Gold et al. 1998). Similar to vertebrates, *C. elegans* olfaction also signals through GPCR and G-protein pathways. At least 20 Gα subunits are encoded in the *C. elegans* genome, including the homolog of each of the 4 mammalian Gα families, EGL-30(Gq), GOA-1(Go), GSA-1(Gs) and GPA-12(G12), and another 17 Gα subunits that do not have mammalian orthologs (Jansen, Thijssen et al. 1999). One Gα, ODR-3, mediates the sensory function of the AWA and AWC olfactory sensory neurons. The null mutant of *odr-3* is defective in chemotaxis to most of the attractants that are sensed by AWA and AWC. ODR-3 functions redundantly with another nematode-specific Gα protein GPA-2 to mediate the response of AWC to an olfactory attractant butanone (Roayaie, Crump et al. 1998). In contrast to the AWA and AWC neurons, the AWB olfactory sensory neurons detect repellants, such as 2-nonanone. The loss-of-function mutants of *odr-3* are partially defective in long-range avoidance to 2-nonanone (Troemel, Kimmel et al. 1997), suggesting additional G proteins to mediate AWB sensation. Because each *C. elegans* olfactory sensory neuron can express multiple GPCRs, more than one Gα protein may be employed for signal transduction in individual neurons. In this chapter, I show that EGL-30 acts in AWB to mediate its sensory response and mutations in *egl-30* also impair the sensory response of AWC. Therefore, it is possible that Gqα/EGL-30 is one of the Gα proteins involved in olfactory sensation of AWB and AWC. Intriguingly, the activity of EGL-30 in AWB and AWC is essential for aversive training-enhanced *tph-1* expression in ADF neurons, which receive many

synapses from AWB (White, Southgate et al. 1986). These results implicate the importance of sensory experience during training in the up-regulation of *tph-1* expression. AWB and AWC may perceive either sensory inputs from a training pathogen or internal signals generated by infection; the nature of these sensory cues that elicit AWB and AWC responses and mediate *tph-1* regulation will require further investigation.

Methods

Strains

The strains were cultivated and maintained under standard conditions at 20 degrees (Brenner 1974). The expression of *tph-1* was measured using GR1333 *mgIs71 [Ptph-1::gfp, rol-6(su1006)]* V (Sze, Victor et al. 2000). To measure *tph-1* in different mutant backgrounds, all of the following strains were crossed with GR1333: MT2605 *unc-43(n498n1186)* IV, MT1092 *unc-43(n498)* IV, DA823 *egl-30(ad805)* I, MT1434 *egl-30(n686)* I, CG21 *egl-30(tg26)* I; *him-5*(*e1490*) V, KG1278 *unc-73(ce362)* I, RM2221 *egl-8(md1971)* V, JT734 *goa-1(sa734)* I, MT7929 *unc-13(e51)* I and DA509 *unc-31(e928)* IV. The transgenic strains used in this work include CX10281 *kyEx2373 [Pstr-2::GCaMP2; Punc-122::gfp]*, ZC405 *yxEx274 [Psrh-142::YC3.60; Punc-122::dsred]*, ZC28 *unc-43(n498n1186)* IV; *mgIS71* V; *yxEx12 [Psrh-142::unc-43::mcherry; Punc-122::gfp]*, ZC35 *unc-43(n498n1186)* IV; *yxEx19 [Psrh-142::unc-43::mcherry; Punc-122::gfp]*, ZC929 *unc-43(n498n1186)* IV; *yxEx334 [Psrh-142::unc-43::gfp; Punc-122::gfp]*, ZC949 *unc-43(n498n1186)* IV; *mgIS71* V; *yxEx249 [Psrh-142::unc-43(E108K)::mcherry; Punc-122::gfp]*, ZC974 *egl-30(ad805)* I; *mgIS71*V; *yxEx369 [Pstr-1::egl-30::mcherry; Podr-1::egl-30::mcherry; Punc-122::gfp]*, ZC1240 *egl-30(n686)* I; *mgIS71* V; *yxEx543[Pstr-1::egl-30::mcherry; Podr-1::egl-30::mcherry; Punc-122::gfp]*, ZC1145 *yxEx476 [Pstr-1::GCaMP; Punc-122::gfp]*, ZC1493 *egl-30(ad805)* I; *kyEx2373[Pstr-2::GCaMP2; Punc-*

122::gfp], ZC1512 *egl-30(ad805)* I; *yxEx476 [Pstr-1::GCaMP; Punc-122::gfp]*, ZC1584 *egl-30(ad805)* I; *yxEx476 [Pstr-1::GCaMP; Punc-122::gfp]*; *yxEx772 [Pstr-1::egl-30::mcherry; Punc-122::dsred]*, ZC1587 *yxEx775 [Pstr-1::mcherry; Punc-122::dsred; KP326]*, ZC1588 *yxEx776 [Podr-1::mcherry; Punc-122::dsred; KP326]*, ZC1863 *yxEx944 [Psrh-142::mcherry; Punc-122::dsred; KP326]*, ZC947 *egl-30(ad805)* I; *mgIS71* V; *yxEx350 [Pstr-1::egl-30::mcherry; Punc-122::gfp]*, ZC371 *egl-30(ad805)* I; *mgIS71* V; *yxEx246 [Psrh-142::egl-30::mcherry; Punc-122::gfp]*, ZC1391 *mgIS71* V; *yxEx725[Psrh-142::NaChBac::mcherry; Punc-122::gfp]*, ZC1890 *mgIS71V; yxEx960 [Psrh-142::twk-18(cn110)::mcherry*; *Punc-122::dsred]*, ZC2119 *egl-30(ad805)* I; *yxEx249 [Psrh-142::unc-43(E108K)::mcherry; Punc-122::gfp],* ZC2122 *unc-43(n498n1186)* IV; *yxEx249 [Psrh-142::unc-43(E108K)::mcherry; Punc-122::gfp]; yxEx274 [Psrh-142::YC3.60; Punc-122::dsred]*, ZC2153 *itr-1(sa73)* IV; *yxEx274 [Psrh-142::YC3.60; Punc-122::dsred]*, ZC2169 *unc-2(e55)* X; *yxEx274 [Psrh-142::YC3.60; Punc-122::dsred]*, ZC2170 *unc-68(e540)* V; *yxEx274 [Psrh-142::YC3.60; Punc-122::dsred],* ZC 2175 *unc-43(n498n1186)* IV; *yxEx12 [Psrh-142::unc-43::mcherry; Punc-122::gfp]; yxEx274 [Psrh-142::YC3.60; Punc-122::dsred]*. KP326 is a plasmid that contains a *Pegl-30::gfp* transcriptional reporter (Lackner, Nurrish et al. 1999).

Generation of DNA transgenes and transgenic animals

To generate the *Psrh-142::YC3.60* construct, a 4 kb PCR product of the *srh-142* promoter was cloned into Invitrogen pENTR to make an entry vector, which was used in an LR recombination reaction with a pCVG11 destination vector containing Cameleon YC3.60 and *unc-54* 3′UTR to generate the final construct. A PCR product containing the *srh-142* promoter, YC3.60, and *unc-54* 3′UTR was generated from the plasmid and injected at 100 ng/µl. To generate the *Psrh-142::unc-43::mcherry* construct, the mcherry cDNA was used to

replace the *gfp* sequence in pPD95.77 (a gift from Dr. A. Fire, Stanford University School of Medicine, Stanford, CA), and a 4 kb *srh-142* promoter was inserted into the SphI site of the same plasmid to generate *Psrh-142::mcherry. unc-43* cDNA, which was isolated from the clone *yk213a11* (a gift from Dr. Y. Kohara, National Institute of Genetics, Mishima, Shizuoka, Japan), was inserted into *Psrh-142***::***mcherry. Psrh-142::unc-43(E108K)::mcherry* was constructed using a QuickChange Site-Directed Mutagenesis kit (Stratagene) from *Psrh-142::unc-43::mcherry*. To generate the *Psrh-142::twk-18(cn110)::mcherry* and *Psrh-142::NaChBac::mcherry* constructs, the coding region of *twk-18(cn110***)** (a gift from Dr. E. Jorgensen, The University of Utah, HHMI, Salt Lake City, UT) or NaChBac (a gift from Drs. D. Clapham and D. Ren, Harvard Medical School, HHMI, Boston, MA) was amplified with primers engineered with SalI sites and was used to replace the *unc-43* cDNA in the *Psrh-142::unc-43::mcherry* construct. An *egl-30 cDNA* was isolated from the clone *yk587a10* (a gift from Dr. Y. Kohara). To generate *Psrh-142::egl-30***::***mcherry*, a *srh-142* promoter fragment and a PCR product of *egl-30* cDNA were inserted into a *mcherry* vector, which was generated by replacing the *gfp* sequence of pPD95.77 with a *mcherry* coding sequence. The transgenic animals were generated by germline transformation (Mello, Kramer et al. 1991). The transgenes were injected at 25 ng/µl together with a coinjection marker, *Punc-122::gfp* or *Punc-122::dsred* (Miyabayashi, Palfreyman et al. 1999), at 25 ng/µl, unless otherwise noted.

Aversive olfactory training

The animals were trained based on a long-term training procedure as described previously (Zhang, Lu et al. 2005). Briefly, the embryos were extracted and placed on nematode growth medium (NGM) plates. For the naive condition, the NGM plate contained a lawn of *Escherichia coli* OP50. For the standard training, the NGM plate contained a lawn

of *Pseudomonas aeruginosa* PA14 and a small lawn of OP50. For mutants with locomotory defects, L4 animals on the standard training plate were moved to another training plate that was completely covered by PA14 to ensure sufficient training.

Fluorescence Microscopy

The *gfp* images were collected using an Eclipse TE2000U fluorescence inverted microscope (Nikon) with a 40× NA1.3 oil-immersion objective and a CoolSNAP EZ CCD camera; the quantification was performed using NIS-element software and NIH ImageJ. The mean fluorescence intensity was generated for each neuron by subtracting the background intensity from the average fluorescence intensity of the neuron. Multiple worms were measured for each genotype.

Intracellular calcium imaging

The neuronal calcium response of ADF neurons was measured by quantifying the changes in the YFP/CFP ratio of the ratiometric calcium indicator Cameleon YC3.60 (Nagai, Yamada et al. 2004). The neuronal responses of AWB and AWC were measured by quantifying the fluorescence intensity change of the calcium indicator GCaMP (Nakai, Ohkura et al. 2001) or GCaMP2.0 (Tallini, Ohkura et al. 2006). A microfluidic device (Chalasani, Chronis et al. 2007; Chronis, Zimmer et al. 2007) was used to trap the worm and to deliver fluidic stimuli. The switch between the buffer and the bacteria-conditioned medium was controlled by an Automate Scientific Valvebank II Perfusion System (Berkeley, CA). To make the bacteria-conditioned medium, *E. coli* OP50 was cultured in nematode growth medium (NGM) overnight until OD 600 reached 0.25 to 0.30. The bacterial culture was then filtered with a 0.22μm PES membrane filter. ND2 files of fluorescence images were collected using a CoolSNAP EZ CCD camera installed on a Nikon Eclipse Ti-U inverted Microscope with a 40x NA 1.3 oil immersion objective. The

sampling frequency was 10 Hz. The fluorescence intensity was quantified by analyzing the ND2 files with a customized Matlab script that tracked and measured the average intensity of a designated number of brightest pixels in the selected region of interest (ROI) in each image throughout the image series, which was then subtracted by a background signal of a nearby region. To correct the photo-bleaching in the Cameleon signal, the signal was fitted into a bi-exponential decay function and the corrected signal was the ratio of the recorded signal over the decay function. To calculate the change in signal, the average signal in a 4-second window before the switch of the stimuli was set as the baseline F_0. The percentage change relative to F_0 was calculated and plotted.

Slow-killing Assay

Slow-killing assays were performed using a procedure similar to the one described previously (Kawli, Wu et al. 2010). Briefly, the worms were made sterile by feeding on the***pos-1*** RNAi to minimize the effect of progeny hatching inside hermaphrodite bodies during the assays. The killing assays were performed on a 3.5 cm NGM plate covered with PA14 at 25°C. The worm corpses were picked out and scored every 8–10 h, and the worms that had died on the wall of the Petri dish were censored.

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Chapter 3

Progress towards understanding of the neuroplasticity underlying aversive learning

3-I: Probing the link between *tph-1* transcription regulation and learning

Results

Short-term training induces *tph-1* transcription up-regulation

C. elegans can learn to avoid the odor of the pathogenic bacteria PA14 after training with either of the two related but distinct paradigms. One of them is the long-term training paradigm described in Chapter 2. In this paradigm, worms grow up on an OP50 lawn, but have been exposed to the odor of PA14 since they hatch from the embryos. Before L4 larvae stage, the worms only occasionally crawl to the PA14 lawn. Around the L4 larvae stage, the worms will consume all of the OP50 bacteria. After this time point, all the worms will crawl to the PA14 lawn and receive the aversive training. The other paradigm is the short-term training paradigm, in which worms are raised on OP50 plate until they reach adulthood. The young adults are transferred from the OP50 plate to a PA14 training plate and trained on PA14 lawn for 4 to 6 hours.

Different from the *tph-1* transcriptional regulation in the long-term training paradigm described in Chapter 2, we cannot detect a significant increase of *tph-1*::*gfp* level when measured with the strain GR1333 as the reporter in the short-term paradigm (Figure 3.1A). However, with a single copy reporter of *tph-1*::*gfp*, which is more sensitive, a significant but small increase in *tph-1* level was observed after the short-term training (Figure 3.1 B, Dr. Xiaodong Zhang's data with strain QL302).

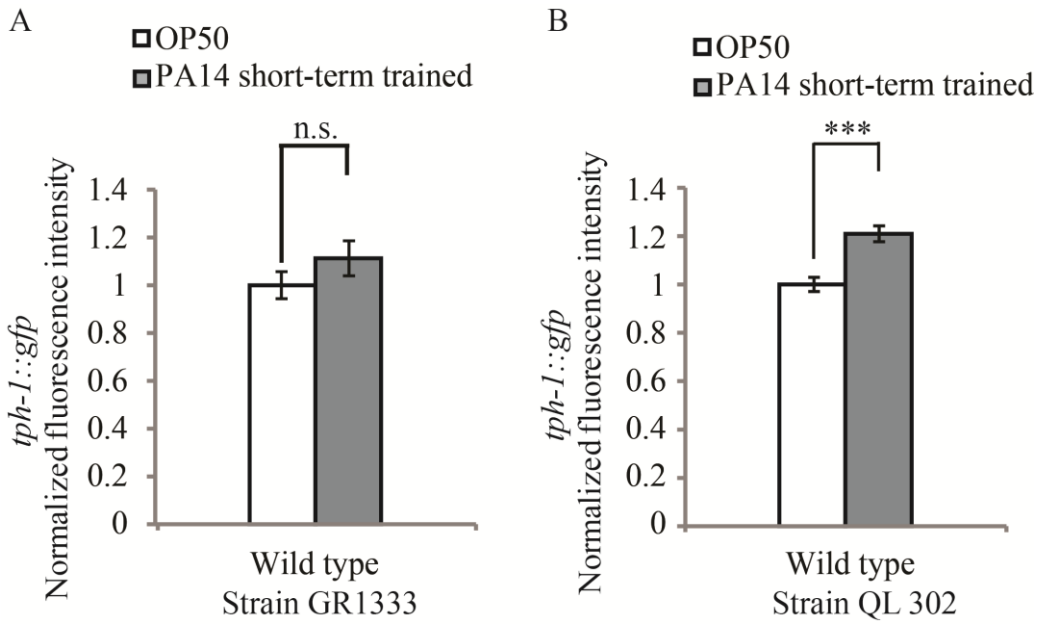

Figure 3.1 Short-term PA14 training induced ADF *tph-1* mildly but significantly.

A*, *tph-1*::*gfp level measured in the multiple copy reporter strain is not up-regulated in worms trained with PA14 in the short-term training procedure. (n.s. not significant in Student's *t* test, n = 25 worms for each group). ***B,*** ***tph-1*::*gfp*** level measured in the single-copy reporter strain is up-regulated in worms trained with PA14 using the short-term training procedure. (*** $p < 0.001$ for Student's *t* test, n = 37 worms for each group).

The *gacA* mutant of the *Pseudomonas aeruginosa* PA14 up-regulates *tph-1* transcription in the long-term aversive training, and induce mild olfactory preference changes

Pseudomonas aeruginosa PA14 *gacA* is a mutant strain of PA14 that was identified with a severely reduced killing rate of *C. elegans* under the slow killing condition (Tan, Rahme et al. 1999). The accumulation of wild type PA14 in the intestine of the worms can be detected around 48 hours after infection, while the accumulation of PA14 *gacA* in the intestine cannot be detected until after 72 hours (Tan, Mahajan-Miklos et al. 1999). GacA is a virulence factor in the GacS/GacA two-component system, which is conserved in gram-negative bacteria. GacS is a sensor kinase that senses certain environmental signal and GacA is the response regulator that triggers the transcription of downstream target genes (Heeb and Haas 2001). GacS/GacA system controls the production of cyanide, lipase, pyocyanin, and the autoinducer *N*-butyrylhomoserine lactone (BHL) in *Pseudomonas aeruginosa*, although there seems to be signaling molecules that are regulated independently of GacA, such as N-oxododecanoyl-L-homoserine lactone and toxin A (Reimmann, Beyeler et al. 1997).

To seek the components in PA14 that cause *tph-1* transcription up-regulation and to test if *tph-1* transcription up-regulation alone is sufficient to induce learning, I measured *tph-1*::*gfp* level and quantified learning behavior after long-term training with PA14 *gacA* strain. Surprisingly, *tph-1* level is highly up-regulated by the *gacA* strain (Figure 3.2 A), but worms did not avoid the odor of *gacA* after training (Figure 3.2 B). Notice that the larger the choice index, the stronger the worms prefer PA14 *gacA* odor. There is a small but significant difference in the choice index between the control worms and the worms trained by PA14 *gacA*, suggesting that the worms trained with *gacA* prefer *gacA* less than the control worms.

While these results are consistent with the possibility that increased *tph-1* expression in ADF leads to decreased olfactory preference for the training bacteria, the observed behavior change induced by *gacA* mutant is relatively small. These findings together with our previous results on PA14-induced changes in *tph-1* transcription and learning behavior suggest that up-regulation of *tph-1* transcription in ADF alone is not sufficient to induce a prominent decrease in olfactory preference; some other unknown factors are also required.

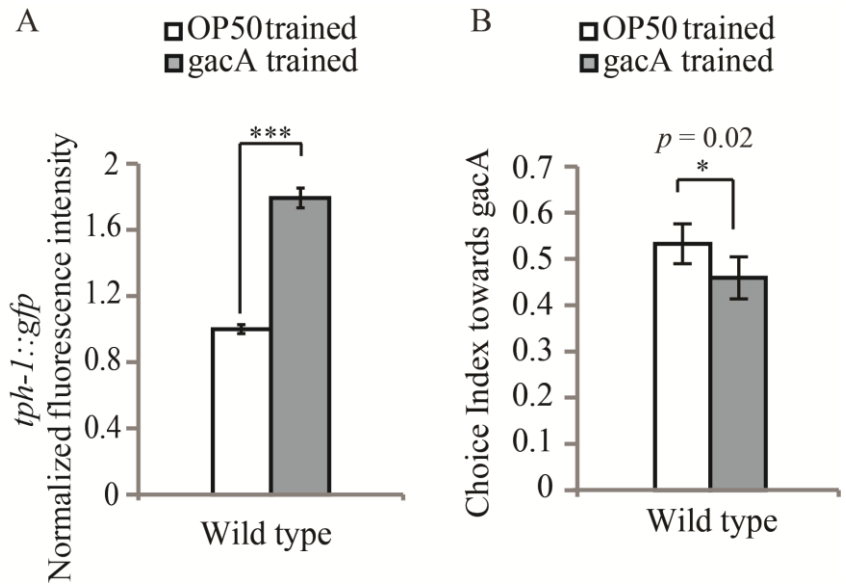

Figure 3.2 PA14 *gacA* strain induces ADF *tph-1* transcriptional up-regulation and a mild decrease in olfactory preference for *gacA*.

A, *tph-1*::*gfp* level is up-regulated in worms trained with PA14 *gacA* in the long-term training procedure ($p < 0.001$ for Student's *t* test, n = 101 worms for each group).

***B*,** Choice index data from the droplet assays after long-term training on *gacA* ($p = 0.02$ one-tailed paired Student's *t* test, n = 11 assays for each group). *gacA* trained worms prefer the odor of *gacA* less compared with the control worms.

What is the factor in PA14 that induces *tph-1* transcription? One of the possibilities is that the smell of PA14 without actual infection is sufficient to induce *tph-1* transcription. To test this hypothesis, I raised the worms on a plate with a small OP50 lawn similar to the OP50 lawn on the long-term training plate. However, instead of covering the other half of the plate with PA14, I covered the plate with a whole plate of PA14 on the top as the lid (Figure 3.3 A). This way the worms are exposed to the odor of PA14 since larvae stages, but would not be infected by the pathogenic bacteria. When compared with worms grown on the same plate with a small OP50 lawn, but covered by a blank plate, I did not observe an induction of *tph-1* transcription (Figure 3.3 B). This result argues that the smell of PA14 alone is not sufficient to induce *tph-1* transcription up-regulation.

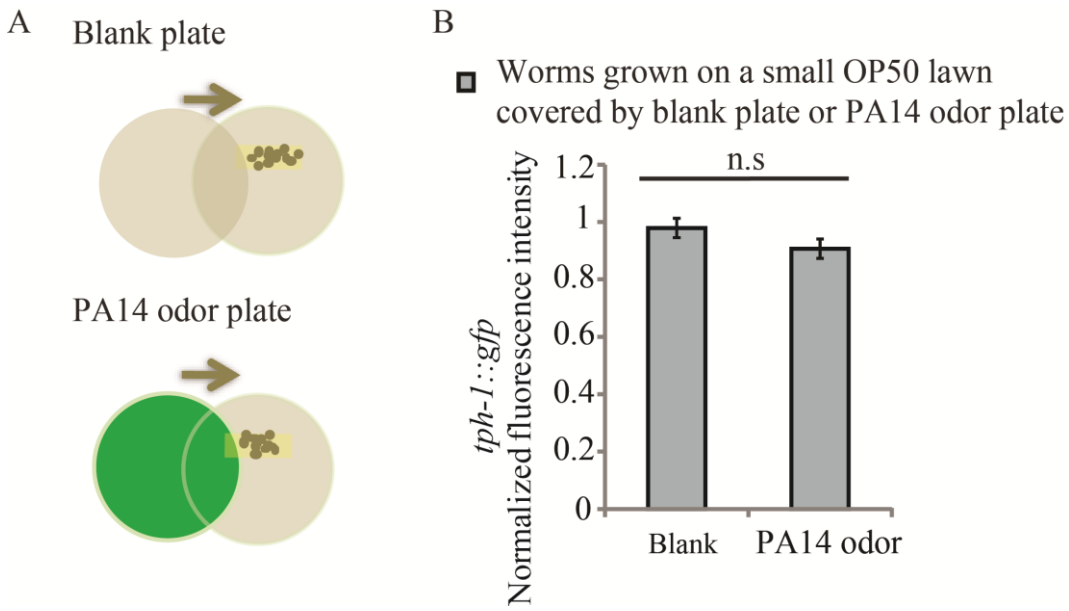

Figure 3.3 The smell of PA14 alone cannot up-regulate *tph-1* transcription.

***A*,** A plate with a small lawn of OP50 similar to the long-term training plate is covered with a blank plate or a PA14 plate. Embryos were developed on the small OP50 lawn. ***B*,** *tph-1*::*gfp* level of worms from the plate covered by PA14 lawn is not significantly different from that of worms from the plate covered by the blank plate. (n.s $p = 0.13$ Student's t test, n = 60 for the blank group, n = 59 for the PA14 odor group).

In terms of the component of PA14 that induces *tph-1* transcription, the other possibility is the weak virulence in *gacA* mutant. Infection response gene 1, *irg-1,* is a gene with unknown functions, but is induced by PA14 infection via a pathway that is independent of known immunity pathways, such as p38 MAPK pathway and leucine-rich repeat G protein-coupled receptor FSHR-1 (Estes, Dunbar et al. 2010). It was reported that the infection by PA14 *gacA* for 8 to 20 hours cannot induce *irg-1::gfp* in the slow killing assay (Estes, Dunbar et al. 2010). However, when worms are exposed to PA14 *gacA* using the long-term procedure, *irg-1::gfp* is up-regulated similar to the level of *irg-1::gfp* in the PA14 infected animals (Figure 3.4). All together, these results suggest that the remaining pathogenicity in PA14 *gacA* is sufficient to induce *tph-1* transcription up-regulation, but not sufficient to induce a strong olfactory aversion.

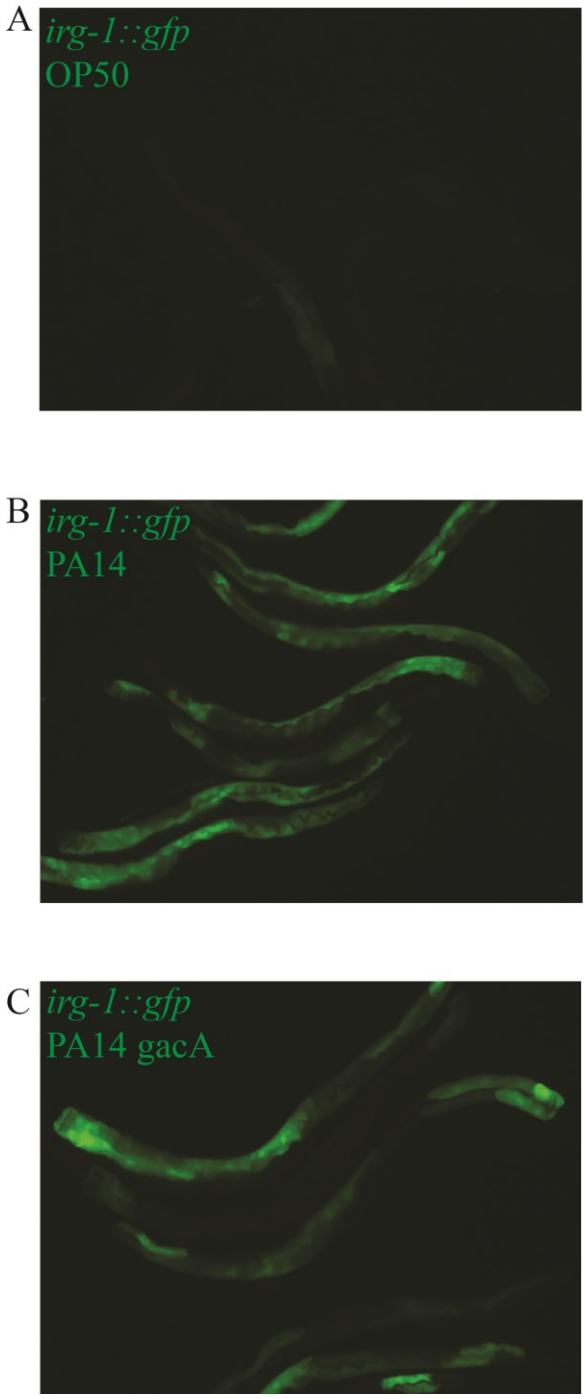

Figure 3.4 PA14 *gacA* infection up-regulates the expression of *irg-1::gfp* in the intestine after long-term training. ***A*****,** *irg-1::gfp* signal from worms grown on OP50 plate. ***B***, *irg-1::gfp* induction after PA14 long-term training. ***C*****,** *irg-1::gfp* induction after PA14 *gacA* long-term training.

Up-regulating *tph-1* transcription in ADF alone with the NaChBac channel is not sufficient to induce olfactory avoidance.

Based on results in Chapter 2, both the ADF calcium activity and *tph-1* transcription level are increased by PA14 training. Furthermore, the expression of the NaChBac channel in the ADF neurons can up-regulate the level of *tph-1* transcription without training. Thus I asked whether it is possible to alter the behavior of the worms by expressing the NaChBac transgene in the ADF neurons. To answer this question, I tested the naive choice index in ADF::NaChBac transgenic worms, to see if the naive preference can be altered without training. I did not observe any difference in the naive preferences between transgenic and non-transgenic worms (Figure 3.5 A).

Next, I tested if the expression of NaChBac could speed up the learning process. To test this hypothesis, I performed time-course learning assay, in which I tested the learning behavior in the droplet assay after the worms have been trained for 1, 2 or 4 hours. Again, the data showed that the expression of the channel does not speed up the learning process (Figure 3.5 B).

All together, these data suggest that the expression of the NaChBac channel in the ADF neurons can up-regulate the *tph-1* transcription level, but is not sufficient to alter the olfactory preference.

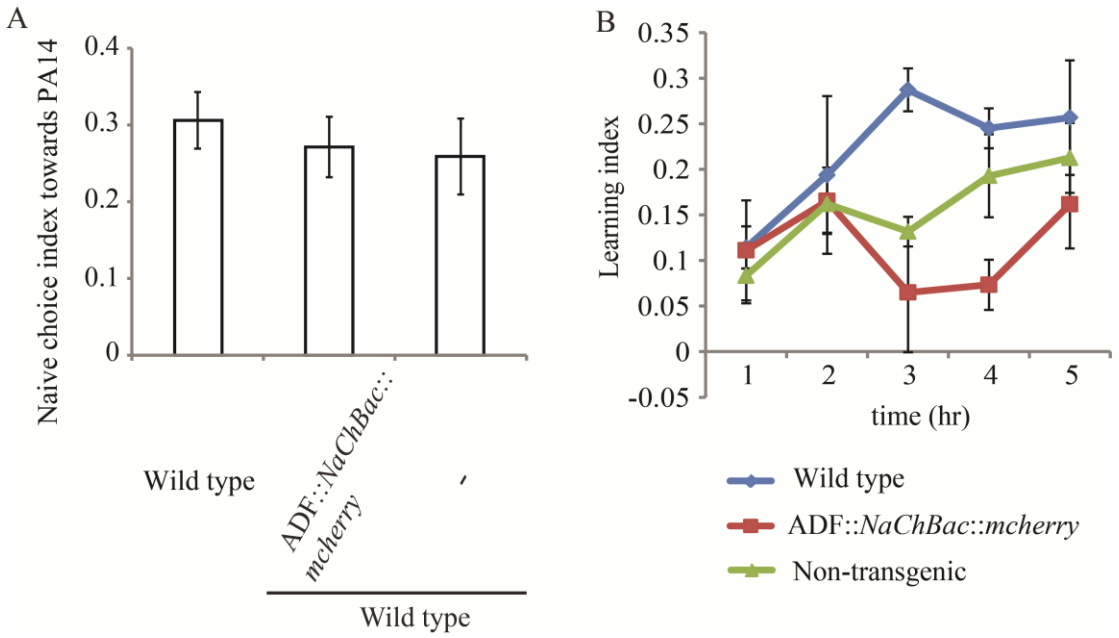

Figure 3.5 Expression of the NaChBac channel in the ADF neurons is not sufficient to induce avoidance without pathogenic bacteria training.

***A*,** Expression of NaChBac in the ADF neurons does not change the worms' naive preference. (n = 17 assays). ***B*,** Time course measurement showed the expression of NaChBac in the ADF neurons did not speed up the learning behavior.

Reducing ADF activity with *twk-18(gf)* is not potent enough to block learning.

As described in Chapter 2, inhibition of ADF with *twk-18(cn110)* can suppress the up-regulation of *tph-1* transcription after PA14 training. To test if the expression of *twk-18(cn110)* in the ADF neurons can also block learning, ADF::*twk-18(cn110)* transgenic worms were tested in the droplet assay after the long-term training. These worms can still learn to avoid the smell of PA14 after training (Figure 3.6). This result shows that the inhibition of the ADF neurons with *twk-18(cn110)* is not sufficient to block learning.

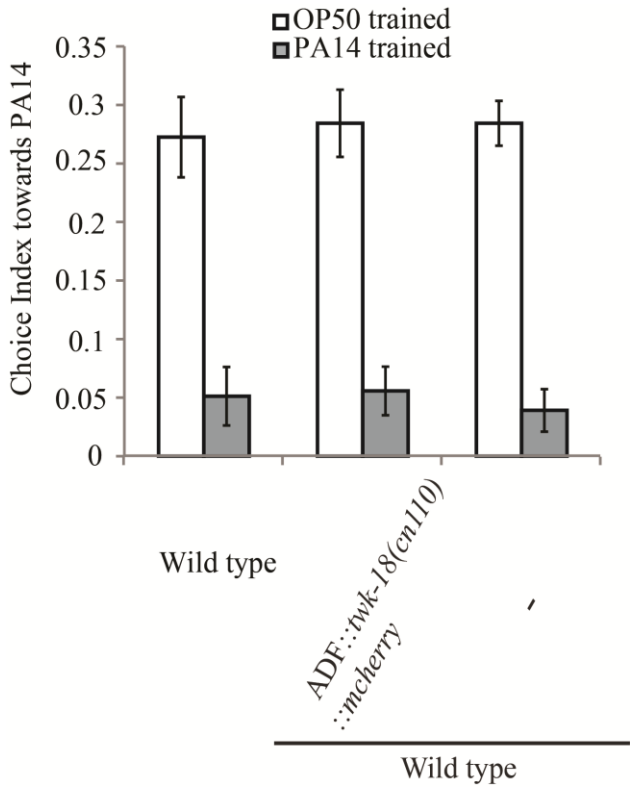

Figure 3.6 Choice index data from the droplet assay of the *twk-18(cn110)* expressing worms after long-term training. n = 23 assays. There is no significant difference by genotype tested with 2-way Anova.

Discussions

Manipulation of ADF *tph-1* transcription level alone is not sufficient to alter the learning

I tested the correlation between *tph-1* transcriptional up-regulation induced by the pathogenic bacteria training and the learning behavior induced by the pathogenic bacteria training from 4 aspects: First, the short-term training procedure is strong enough to induce the olfactory aversive learning behavior, meanwhile it has a small but significant effect on ADF *tph-1* transcription up-regulation; Second, the PA14 *gacA* mutant significantly up-regulates the ADF *tph-1* transcription level, but the animals do not avoid the smell of *gacA*; Third, the expression of the bacterially derived sodium channel NaChBac in the ADF neurons increased the ADF *tph-1* level significantly even in the absence of training, but this manipulation did not alter the behavior of the animals; Fourth, the expression of the gain-of-function potassium channel *twk-18(cn110)* in the ADF neurons suppressed the ADF *tph-1* up-regulation induced by the PA14 training, but did not block the learning behavior.

All together, these data suggest that: First, the transcription up-regulation of *tph-1* alone is not sufficient to induce the olfactory aversive learning behavior. There are other signals in the nervous system that is needed for the learning behavior (Zhang and Zhang 2012; Chen, Hendricks et al. 2013) and these signals cannot be triggered by serotonin signal alone. Second, the 2-folds up-regulation of ADF *tph-1* transcription induced by the training is not necessary for the olfactory aversive learning behavior. Given that serotonin from the ADF neurons is essential for the learning and exogenous serotonin can speed up the learning process (Zhang, Lu et al. 2005), and our observation that the *gacA* training has a small but significant effect in reducing the olfactory preference for the smell of *gacA* (Figure 3.2), the enhanced serotonin signaling

from the ADF neurons may still contribute to the learning to avoid *gacA* in this context. With a different reagent that can suppress ADF neuronal activity even more and can abolish the *tph-1* transcription up-regulation even when measured with the single copy reporter, a learning defect may be detected to demonstrate the necessity of increased serotonin signaling for the behavior.

Critical period for *tph-1* up-regulation: increased neuronal plasticity or decreased stress defense in *C. elegans* larvae?

The long-term training procedure induces *tph-1* transcription, and the extent of up-regulation is reduced significantly in the short-term training procedure. If the training started around L4 stage, in the condition that the worms are grown on an OP50 plate until L4 stage then picked to a PA14 plate, the up-regulation of *tph-1* transcription becomes instable. A similar observation was reported in the *tph-1* transcription up-regulation induced by raised temperature (Estevez, Estevez et al. 2004). In this scenario, the developmental stage from L2 to L3 seems to be critical for the ADF *tph-1* up-regulation. If the worms born in 15 degree are moved to 25 degree before this critical stage, ADF *tph-1* will be up-regulated. If worms born in 25 degree are moved to 15 degree after this stage, ADF *tph-1* level will stay high (Figure 3.7). This phenomenon is very intriguing. In both scenarios, ADF *tph-1* levels can be up-regulated by the specific treatment, but the treatment must be applied before certain critical stage to exert this effect. Is it possible that in the larvae, there is an increased neuronal plasticity? Or is it because the larvae have a low ability for stress defense? Future studies will be needed to address these questions.

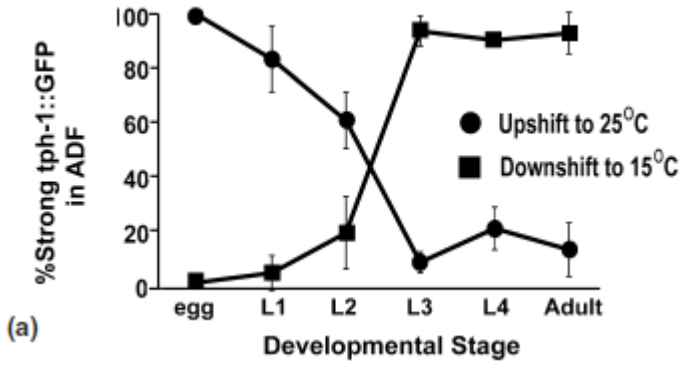

Figure 3.7 Critical window for ADF *tph-1* regulation by raised temperature. (Estevez, Estevez et al. 2004)

What signals up-regulate the *tph-1* transcription?

Pseudomonas aeruginosa PA14 releases a range of metabolites and virulence factors to the environment. It is intriguing to ask which factors induce *tph-1* transcription up-regulation in *C. elegans*. When the worms are exposed to the smell of PA14 since hatch, but do not actually become infected by PA14, the *tph-1* transcription is not induced (Figure 3.3). This argues that the smell of PA14 alone is not sufficient for the transcription up-regulation, and infection by the pathogenic bacteria is required for the transcription up-regulation. Since training with PA14 *gacA* can also induce *tph-1* transcription, it suggests that the remaining virulence in PA14 *gacA* in the long-term infection is sufficient to induce *tph-1* transcription.

On the other hand, the AWB and AWC calcium imaging in *egl-30* loss of function background suggests that Gq *egl-30* mediates AWB and AWC sensory response to induce *tph-1*

transcription. This suggests that certain signal sensed by the olfactory sensory neurons is also required for the *tph-1* transcription. These olfactory stimuli could be certain secondary metabolites released by PA14 that are independent of *gacA* control. It is also possible that these stimuli are certain physiological signaling molecules released by the infected worm body.

A related question is what molecules released by the pathogenic bacteria PA14 make the odor of PA14 appetitive to the worm. Since PA14 *gacA* mutant is still strongly attractive to the worms when compared with the odor of *E. Coli* OP50, the main attractive odor components seem to be independent of *gacA*. Dr. Heonick Ha in the lab tried several chemicals that exist in PA14 smell such as benzaldhyde (unpublished data), but none of these odors can replace the odor of PA14 in the olfactory preference test in the learning assay. Interestingly, in a recent study that analyzed the naive preference and conditioned learning in hawkmoth *Manduca sexta*, the authors trapped and identified oxygenated aromatic compounds, including methyl benzoate, benzylalcohol and benzaldhyde, as the main components of the floral volatile organic compounds (VOCs) of hawkmoth-visited flowers, using dynamic sorption methods and gas chromatography with linked mass spectrometry (GCMS) (Riffell, Lei et al. 2013). Given the complexity of PA14 metabolites, a detailed chemical analysis of PA14 odor similar to the hawkmoth study would be helpful to answer these questions including the chemical components of PA14 that induce *tph-1* transcription or the chemical components of PA14 that render it attractive to the worms.

What are the physiological consequences of ADF *tph-1* transcriptional up-regulation?

Based on literatures related to the function of serotonin from the ADF neurons, it seems that the enhanced serotonin signaling from the ADF neurons can exert various modulatory effects on the animal behaviors in different contexts. For instance, the results from previous studies suggest that high level of ADF *tph-1* promotes strong hyperoxia avoidance (Chang, Chronis et al. 2006) and increases pharyngeal pumping rate in presence of food (Cunningham, Hua et al. 2012). Another study suggested that increased serotonin release further increases pharyngeal pumping in presence of familiar food (Song, Faumont et al. 2013).

It is worth noticing these studies generated opposite implications on the effect of food signal on ADF *tph-1* expression. The presence of food suppresses hyperoxia avoidance, and this modulatory effect is absent in *tph-1* mutant. The authors suggested that ADF *tph-1* endogenous regulation is required to convey the food signal (Chang, Chronis et al. 2006). Thus, all the data from this oxygen sensation study argue that the food signal down-regulates *tph-1* expression in ADF neurons. This is opposite to the implications from the pharyngeal pumping studies (Cunningham et al., 2012, Song et al., 2013). Since food signal or familiar food signal increases pharyngeal pumping and these effects are depends on ADF *tph-1*, these studies argue that food signal up-regulates ADF *tph-1* expression. These two contrary arguments are based on analysis of behavioral phenotypes of mutants, because authors of these studies did not quantify the changes of the ADF *tph-1* expression induced by the food signal directly. The familiar food signal indeed increased serotonin release from the ADF neurons and this could be independently regulated from increased *tph-1* expression (Song et al., 2013).

In the context of olfactory aversive training with the pathogenic bacteria, clearly *tph-1* transcription is enhanced by the aversive experience (Zhang, Lu et al. 2005). It is not quite intuitive if increased *tph-1* expression leads to enhanced pharyngeal pumping on the pathogenic bacteria or enhanced hyperoxia avoidance after pathogenic bacteria training. Further characterization of the trained animals' behaviors will help to test these possibilities.

Previous literatures also suggest ADF *tph-1* expression may regulate stress response of the worms by inhibiting the nuclear translocation of FOXO transcription factor DAF-16 (Liang, Moussaif et al. 2006). Insulin growth factors (IGF) signaling inhibits FOXO activity; while activation of FOXO transcription factors leads to nuclear translocation of these transcription factors and in turn elevates expression of stress response genes and inhibits development and reproduction. In *C. elegans*, nuclear accumulation of DAF-16 leads to developmental arrest. It was reported that DAF-16 accumulates in nuclei of *tph-1* mutant. Expression of *tph-1* in the ADF neurons can suppress this nuclear accumulation, while expression of *tph-1* in the NSM neurons alone cannot suppress the DAF-16 nuclear accumulation (Liang, Moussaif et al. 2006). Based on these data, it was proposed that increased ADF *tph-1* may inhibit nuclei accumulation of DAF-16 and promote cytoplasmic distribution of DAF-16. To test this hypothesis, starvation for 4 hours is used as a stress stimulus. This stress stimulus decreases ADF *tph-1* without altering NSM *tph-1* and induces DAF-16 nuclei accumulation (Liang, Moussaif et al. 2006). Given that DAF-16 accumulation induced by starvation does not depend on *tph-1*, the authors suggested food signal may stimulate ADF serotonin synthesis and promotes DAF-16 cytoplasmic distribution. Consistent with the hypothesis, DAF-16 did not accumulate in nuclei after 5 hours PA14 infection (Liang, Moussaif et al. 2006), if we expect 5 hours PA14 infection will up-regulate ADF *tph-1*. However, inconsistent with this hypothesis, dauers display increased ADF *tph-1* and

increased *daf-16* nuclei accumulation (Moussaif and Sze 2009). Enhanced ADF serotonin cannot promote cytoplasmic distribution of DAF-16 when dauer is used as a stress stimulus. The third instance is increased temperature. Raising temperature to 26 degree increases ADF *tph-1* and also increases DAF-16 nuclei accumulation (Estevez et al., 2004; Liang et al., 2006). Enhanced ADF serotonin cannot promote cytoplasmic distribution of DAF-16 when heat is used as a stress stimulus. The regulation of DAF-16 localization is a complex process and is not directly correlated with the expression level of ADF *tph-1*.

In summary, the ADF *tph-1* level can be regulated by a variety of environmental stimuli and serotonin from ADF neurons can modulate a variety of animal behaviors. Current data generated inconsistent implications on the ADF *tph-1* level and its effect on behaviors. It seems the modulatory effects of different ADF *tph-1* levels are highly context dependent and behavior dependent. More research efforts would be required to reach a consensus model.

Methods

Strains

The strains characterized in this chapter including: GR1333 *mgIs71* **[***tph-1::gfp, rol-6(su1006)***]** V (Sze, Victor et al. 2000), AU 133 [*Pirg-1::gfp*] (Estes, Dunbar et al. 2010), QL 302 [*Ptph-1::gfp*] single copy reporter, ZC1491 yxEx 726 [*Psrh-142::NaChBac::mcherry*], ZC1914 yxEx 960 [*Psrh-142::twk-18(cn110::mcherry*].

Long-term and short-term aversive olfactory training

Long-term training procedure is essentially the training procedure described in Chapter 2. Briefly, the embryos were extracted and placed on nematode growth medium (NGM) plates. For the naive condition, the NGM plate contained a lawn of *Escherichia coli* OP50 grown in 27

degree incubator for 2 days. For the standard training, the NGM plate contained half a plate *Pseudomonas aeruginosa* PA14 and a small lawn of OP50 grown in 27 degree incubator for 2 days. For mutants with locomotory defects, L4 animals on the standard training plate were moved to another training plate that was completely covered by PA14 to ensure sufficient training.

Short-term training started from young adult worms. These worms were grown on *Escherichia coli* OP50 plate until adulthood. Then they were picked to a plate covered by PA14 for 4 to 6 hours before the assay.

Microdroplet assay to test learning

Microdroplet assay was performed as described in the previous study (Ha, Hendricks et al. 2010). Basically, 12 microdroplets (2 μl each) of nematode growth medium (NGM) buffer were dropped on a sapphire window (Swiss Jewel Company) in each assay. Each worm was washed in NGM buffer and then picked into each droplet. The window was placed in an enclosed chamber, where the animals were exposed to the alternative air stream of *E. coli* OP50 and *P. aeruginosa* PA14. The air stream was odorized by passage through liquid cultures of bacteria that were shaked overnight at 26°C in NGM medium. The two alternative stimuli were automatically switched by using solenoid valves controlled by LabVIEW (National Instruments, Austin, TX). Images of swimming animals were recorded by a CCD camera at 10 Hz. In each experiment, animals were subjected to 12 successive cycles of alternating 30 s exposure to each odor. The temperature of the sapphire window and the chamber was maintained at 23°C using a temperature-controlled circulating water bath. The number of Ω bend animal performed during the exposure to each odor was analyzed using customized script written in MATLAB

(MathWorks, Natick, MA). The software captured each worm out of the frame by a threshold, and then determined the eccentricity of an ellipse with the same second moments as the region. Since the eccentricity of a worm undergoing an Ω bend (which is roughly circular) is smaller than that of a worm exhibiting forward swimming (which is roughly linear), the script counted one Ω bend if the eccentricity fell below a threshold value, which was set by measuring the eccentricity in a large number of manually detected Ω bends. The turning rate of an assay is calculated from as the mean value for three to six animals in the assay. Finally, the choice index was calculated from the turning rate.

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3-II: Plasticity in the motor program: the locomotory neural circuit for head swing

Introduction

One of the questions remained to be answered is how serotonin modulates downstream neurons for the learning behavior. From analysis of serotonin receptors, it was shown the *mod-1* serotonin-gated chloride channel in the interneurons AIY and AIB is required for the learning behavior (Zhang, Lu et al. 2005). With the laser ablation and droplet assay approach, a neural circuit regulating naive and trained preference for bacteria was mapped (Ha, Hendricks et al. 2010). In this neural circuit, ablation of the *mod-1* expressing AIY neurons or AIB neurons only reduced the naive choice index to half of the wild type level, while ablating both AIY and AIB abolished the naive choice index and thus disrupted learning (Ha, Hendricks et al. 2010). Besides AIY and AIB, interneuron RIA may also be regulated by serotonin from ADF neurons. Moreover, motor neurons RIM, SMD are modulated by interneurons RIA to implement the learning behavior, since ablation of RIM or SMD only disrupted the trained choice index, without affecting the naive choice index (Ha, Hendricks et al. 2010).

In the droplet assay, the change of choice index can be explained by the motor neuron's effect in regulating turning frequency. While during the plate assay, the navigation strategy can be even more complicated, including both klinokinesis, which is characterized by the frequency of turning, and klinotaxis, which is characterized by curving rate bias (Pierce-Shimomura, Morse et al. 1999; Yoshida, Hirotsu et al. 2012). Before we understand the behavioral plasticity regulated by these motor neurons, one way to dissect the circuit is to understand how these motor neurons modulate animals' navigation on plate.

Results

Trained animals display a deeper whole-body undulation

In the neural network mapped with laser ablation approach for the olfactory aversive learning behavior, RIA neurons were identified to be essential for the behavior (Ha, Hendricks et al. 2010). The calcium signaling in RIA neurons displays two components: the global synchronized calcium activity and the compartmentalized calcium activity (Hendricks, Ha et al. 2012). RIA neurons have reciprocal connections with head motor neurons and encode head movement to regulate the whole-body undulation of the animal (Hendricks, Ha et al. 2012). In *gar-3(gk305)* mutant, where the feedback signal conveyed by acetylcholine from SMD to RIA neurons is blocked, the correlation between RIA compartmentalized calcium activity and head movement is disrupted and worms display a deeper body undulation in their locomotion. This locomotory defect of *gar-3(gk305)* mutant is restored when *gar-3* cDNA is reconstituted in RIA neurons (Hendricks, Ha et al. 2012). Given these observations, together with my collaborator Yu Shen, we asked if the pathogenic bacterial training modulates the whole-body undulation during locomotion. To answer this question, we recorded the locomotion of worms on blank NGM plates with our worm tracking system and measured the body undulation in naive and trained animals. We found that trained worms display a significantly smaller aspect ratio, which indicates a deeper body undulation (Figure 3.8 A).

Trained worms in general have a smaller body size compared with the naive worms. To ensure the deeper body undulation is not due to the smaller body size, we compared the ratio aspect of young adult worms grown from the naive condition, which have a similar body size as

trained worms, with that of naive and trained worms. We observed that the young adult worms display a similar undulation as that of the naive animals (Figure 3.8 B).

Pathogenic bacteria *Pseudomonas aeruginosa* PA14 has a different texture compared with *E.Coli* OP50. To test if the undulation effect of training is due to the texture modulation of worm locomotion associated with different strains of bacteria rather than pathogenic infection per se, we also tested if PA14 *gacA* training can change the body undulation. We observed that *gacA* trained animals do not exhibit a significantly different body undulation from that of the *E. Coli* OP50 trained animals (Figure 3.8 C). Together, all these data suggest that the pathogenic bacteria infection changed the body undulation of worms during crawling on the plate.

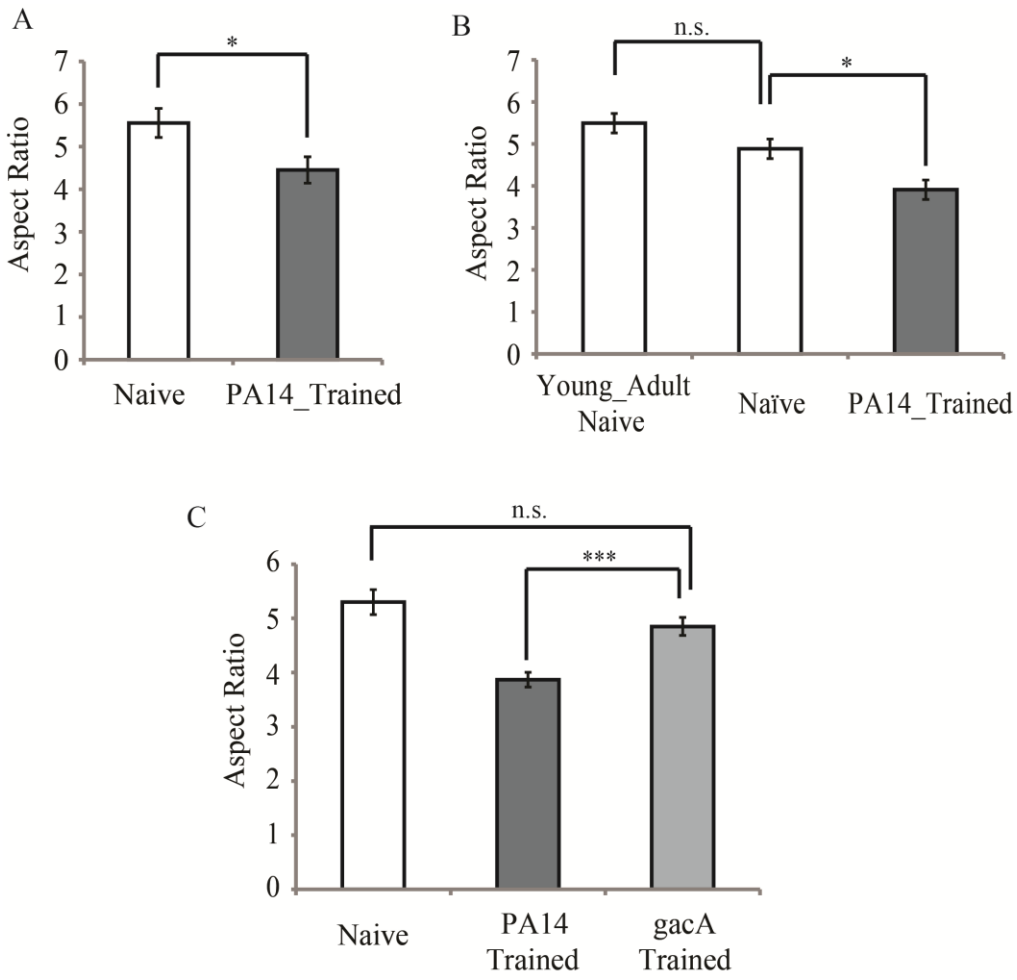

Figure 3.8 The undulation during locomotion is enhanced by *Pseudomonas aeruginosa* PA14 training.

***A*,** PA14 trained worms display a smaller aspect ratio than naïve animals, indicating an increased body undulation. (* $p < 0.05$, Student's *t* test, n = 9 animals). ***B*,** Young adult worms with a smaller body size comparable with PA14 trained animals do not display a smaller aspect ratio. (n.s., not significant, * $p < 0.05$, Student's *t* test with Bonferroni correction, $n \geq 9$ per group). ***C*,** *gacA* trained animals do not display the small aspect ratio as that of the PA14 trained animals. (n.s., not significant, *** $p < 0.001$, Student's *t* test with Bonferroni correction, $n \geq 7$ per group).

The increased undulation in trained worms is not relevant to navigation strategy for the learned avoidance

Having observed the increased undulation in PA14 trained animals, we asked if this contributes to the navigation for the trained worms to avoid the smell of the pathogenic bacteria. *C. elegans* has been shown to employ two navigation strategies on the assay plate: klinokinesis, which is characterized by the frequency of turning, and klinotaxis, which is characterized by curving rate bias (Pierce-Shimomura, Morse et al. 1999; Yoshida, Hirotsu et al. 2012). The increased undulation potentially could help the animals to change its direction without making an omega turn.

To test this hypothesis, we measured body undulation in worms with ablated RIA neurons or with RIA synaptic transmission blocked. In Figure 3.9 A, transgenic worms express caspase in RIA, which genetically ablates the neuron. In Figure 3.9 B, TeTx (Tetanus Toxin) is used to block synaptic transmission in RIA neurons. RIA has been shown to be essential for the learning behavior, since RIA ablated worms cannot learn to avoid the smell of the pathogenic bacteria. If worms with defective RIA neurons lose the increased undulation after training, this training dependent modulation may be correlated with the learning behavior and potentially contribute to the display of the learned preference. However, compared with the non-transgenic siblings we did not find any defect in the increased undulation phenotype in RIA ablated worms or RIA synaptic transmission blocked worms, although we are not sure if signaling molecules released by RIA for the learning behavior is through synaptic transmission. These results suggest that the training-dependent modulation of body undulation is not correlated with the learning behavior.

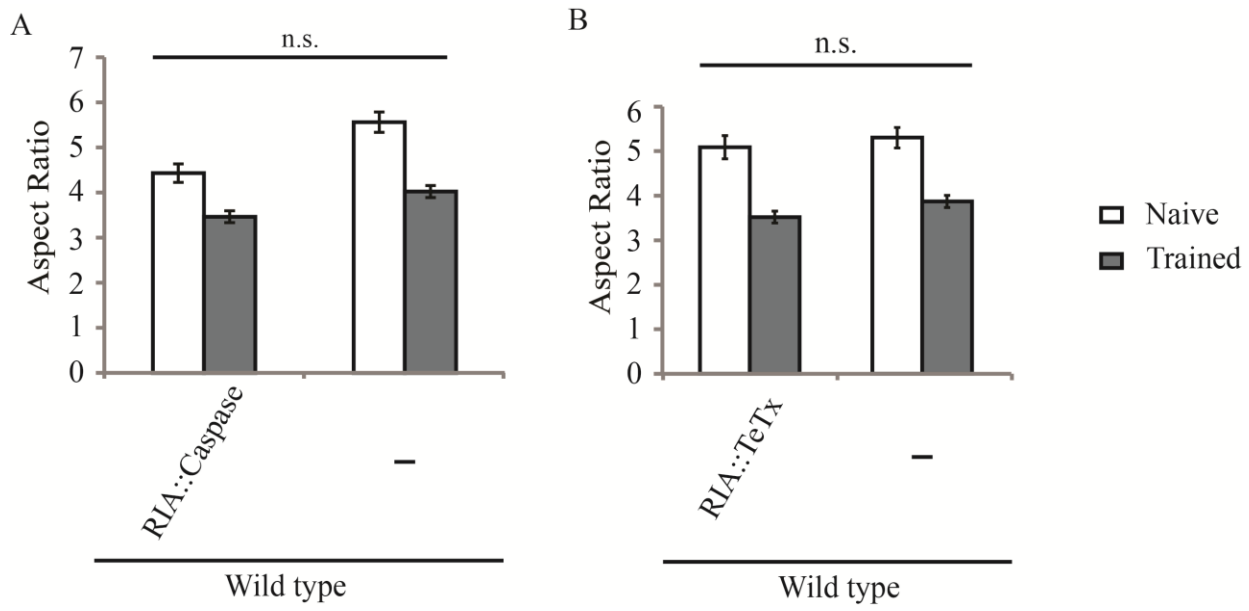

Figure 3.9 The pathogenic bacteria training dependent modulation of body undulation is not correlated with learning.

***A*,** RIA::Caspase expressing worms decrease body aspect ratio as much as the non-transgenic siblings after training. (n.s. not significant, interaction detected by 2-way Anova). ***B*,** RIA::TeTx expressing worms decrease body aspect ratio as much as the non-transgenic siblings after training. (n.s. not significant, interaction detected by 2-way Anova).

Head bending and body curvature are independent

Besides the body curvature, modulation of the extent of head bending may also help the worm to change their forward movement direction during their navigation on the plate. The modulation of head bending is achieved by tuning of the head muscles by the head motor neurons including SMB, SMD, RMD and RME. (White, Southgate et al. 1986White, Southgate et al. 1986). We asked if head bending and body curvature are correlated, for instance, when a mutation of a gene or an ablation of a neuron change the body curvature, whether the same alteration will change the head bending to the same direction.

To test this hypothesis, we measured head curvature and aspect ratio in several different mutants or worms with a specific neuron ablated. In the mutant *gar-3(gk305)*, which the acetylcholine feedback from SMD neuron to RIA neuron is blocked, we observed a decreased aspect ratio, which indicates an increased body curvature. Consistent with the change to the body curvature, we also observed an increased head curvature (Figure 3.10 A, B). Different from the *gar-3(gk305)* mutant, SMB neuron ablated animals also display an increased body curvature, but the head bending only has an insignificant, mild increase (Figure 3.10 C, D). The most striking difference is observed in *unc-25(e156)* strain. *unc-25* is the *C. elegans* homolog of glutamic acid decarboxylase, thus *unc-25(e156)* mutant is defective in GABA synthesis. In this mutant, we did not observe any obvious change in body curvature, but the head bending is significantly increased (Figure 3.10 E, F). All these data suggest that body curvature and head bending are two independent processes that can be modulated independently.

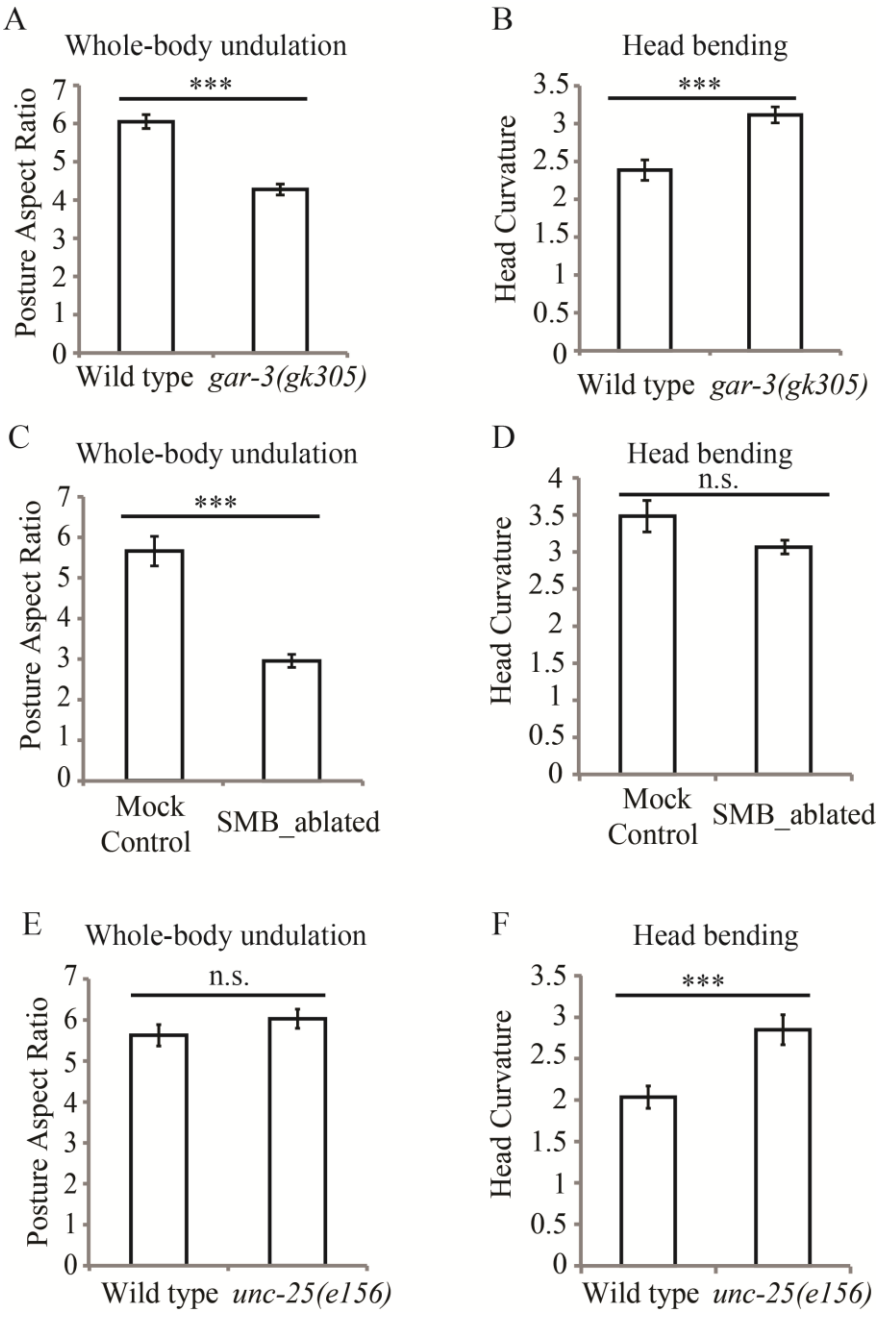

Figure 3.10 The whole-body undulation and head bending are independent modulated behaviors.

Figure 3.10 (continued)

A*, *gar-3(gk305) mutant worms display a significantly decreased posture aspect ratio, indicating an increased body undulation. ***B*, *gar-3(gk305)*** mutant worms display a significantly increased body bending. (n = 8 for each group in ***A*** and ***B***). ***C*,** SMB ablated worms display a significantly decreased posture aspect ratio, indicating an increased body undulation. ***D*,** SMB ablated worms are not significantly different from wild type worms in head bending. (n = 6 for each group in ***C*** and ***D***). ***E*, *unc-25(e156)*** worms are not significantly different from wild type worms in whole body undulation. ***F*, *unc-25(e156)*** worms display a significantly increased body bending. (n = 10 for each group in ***E*** and ***F***, n.s., not significant, *** $p < 0.001$, Student's *t* test)

Methods

Strains

To study the locomotory circuit underlying the animals' navigation strategy, the locomotion of the following strains were tracked and analyzed: ZC1857 *yxIs20* [*Pglr-3::TeTx::mcherry; Punc-122::gfp*], TV2229 [*Pglr-3::caspase; Pglr-3::gfp*], VC657 *gar-3(gk305)* V, CB156 *unc-25(e156)* III.

Tracking and image analysis

For locomotion tracking, well-fed adult animals were briefly washed twice in NGM buffer and picked to a 90-mm NGM agar Petri plate with no food. The entire plate was positioned under a JAI BM-500GE monochrome progressive scan camera (8 bit; 2456(h) x 2058(v) active pixels; 15Hz) with a 60-mm f/2.8D lens (AF Micro-Nikkor; Nikon) for 1 minute before the recording was started. Tracking plates were illuminated by a square array of ultra-red LEDs. Images were captured with a LabVIEW (National Instrument) program at 2 frames per second. For each animal, the frames of backwards and omega turns were excluded and at least 120 frames were analyzed for the forward locomotion. ImageJ software was used to measure the whole-body undulation. First, the midline of the animal's body was extracted and fitted to an ellipse. Then, the aspect ratio of each ellipse was calculated and finally, the average of all frames for each single animal was used as a value for that specific animal. The head curvature was measured with a customized MATLAB script.

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